15. (Amended) A process of claim 12, wherein the linkage is cleavable under acidic, alkaline, neutral or ph tolytic conditions.

16. (Amended) A process of claim 15, wherein the linkage is selected from trityl ether, ester, β-benzoylpropionyl, levulinyl, disulfide and sulfenyl.

26. (Amended) A combinatorial library selected from nucleosides and nucleotides, wherein each compound has from 3 to 10 reactive moieties having blocking groups on the reactive moieties, wherein at least three blocking groups are independently removable under different conditions, thereby allowing selective derivatization after deblocking, and wherein one reactive moiety is utilized for immobilization.

27. (Amended) A combinatorial library according to claim 26, wherein the compounds of the library are oligonucleotides.

28. (Amended) A combinatorial library of claim 26 in which at one or more positions in the sequence a preselected set of building blocks is incorporated.

REMARKS

A check for a three month extension of time ($445 - small entity) accompanies this response. Status as a Small Entity reducing fees to one-half is hereby claimed in this application. Any fees that may be due in connection with this application may be charged to Deposit Account No. 50-1213. If a Petition for extension of time is needed, this paper is to be considered such Petition.

A Change of Address Notification accompanies this amendment.

Claims 4, 11-16 and 26-36 are presently pending in this application. Claims 1-3, 5-10 and 17-25 are cancelled herein without prejudice or disclaimer solely in the interest of advancing the prosecution of this application to allowance. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

Claims 4, 11-16 and 26-28 are amended herein. Basis for the amendments to the claims may be found, for example, in the claims as originally filed. Claims 4 and 26 have been rewritten as independent claims incorporating the limitations of the base claims.

TRAVERSAL OF LACK OF UNITY OBJECTION

Applicant respectfully traverses the Lack of Unity Objection as between Groups I and IV. Applicant has filed a Petition under 37 C.F.R. §1.144 requesting removal of the Lack of Unity Objection as between Groups I and IV, and also requesting removal of the finality of the Objection as being premature. The Petition was filed April 26, 2001, *i.e.*, within 2 months of the mailing date of the instant Office Action.

This application is the U.S. national stage of International Patent Application No. PCT/US97/06509, in accordance with 35 U.S.C. §371. As stated in MPEP 201, national stage applications of international applications are similar to national applications, but there are differences. Among these differences is inapplicability of restriction practice to national stage applications. Restriction practice is applied to national applications, but unity of invention practice is applied to national stage applications (see, MPEP 201 and MPEP 1893.03(d)).

LACK OF UNITY OF INVENTION

Applicant respectfully traverses the Lack of Unity Objection as between Groups I and IV in view of the following remarks. In addition, it is respectfully submitted that the finality of this holding is premature.

Group I, claims 1, 3, 4 and 11-16, is directed to directed to a process of generating a combinatorial set of core molecules of core structure M and a combinatorial set of compounds with core structure M. Group IV, claims 23-36, is directed to a combinatorial set of compounds with core structure M.

PCT Rul 13

This application is the U.S. national stage of International Patent Application No. PCT/US97/06509, in accordance with 35 U.S.C. §371. Applicant notes that no lack of unity objection was raised during either Chapter I or II at the international stage.

As stated in MPEP 201, national stage applications of international applications are similar to national applications, but there are differences. Among these differences is inapplicability of restriction practice to national stage applications. Restriction practice is applied to national applications, but unity of invention practice is applied to national stage applications (see, MPEP 201 and MPEP 1893.03(d)). Therefore, the applicable rule with respect to the instant national stage application is PCT Rule 13.1.

Lack of Unity Standard

When the U.S.Patent Office considers an international application during the national stage, restriction must be based on unity of invention, which is governed by PCT Rule 13 (see MPEP 1893.03(d); Caterpillar Tractor Co. v. Commissioner of Patents and Trademark, 650 F. Supp. 218, 31 USPQ 590 (E.D. Virginia, 1986); In re Caterpillar Tractor Co, 228 USPQ 77). In the Caterpillar cases it was ultimately held that the language in Rule 13.1 "specially adapted" is not to be interpreted as meaning that the process of manufacture can only be used to manufacture the product because this interpretation is in conflict with the PCT Rule, which provides that no national law shall require compliance with requirements relating to the form or contents of the international application different from or additional to those which are provided in the Treaty (Article 27 of the PCT). Thus, the U.S. Patent Office cannot impose requirements that differ from those provided in the Treaty. Since restriction practice differs from and is more restrictive than unity of invention, the unity of invention rules must govern.

Ther fore, it is respectfully submitted, and it appears that th Office has acknowledged, that the rules of unity of invention (PCT Rule 13.1 and 37 C.F.R. §1.475) apply to this application. Rule 13.1 requires that an international application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept.

Groups I and IV do not lack unity under PCT Rule 13

It is respectfully submitted that Groups I and IV relate to a product and a process for the manufacture of said product, and therefore do not lack unity of invention under PCT Rule 13.

Groups I and IV are directed to a process for generating a combinatorial set of molecules of core structure M, and a combinatorial set of compounds with core structure M, respectively. Such groups of claims do not lack unity of invention under PCT Rules 13.1 and 13.2. See 37 CFR §1.475(b):

> An international or a national stage application containing claims to different categories of invention will be considered to have unity of invention if the claims are drawn only to one of the following combinations of categories:
>
> (1) A product and a process specially adapted for the manufacture of said product...

The claims of Group IV are directed to a product (a combinatorial set of compounds with core structure M), and the claims of Group I are directed to a process for the manufacture of the product (a process for generating a combinatorial set of molecules of core structure M). Such Groups of claims do not lack unity of invention, and therefore should be examined in one application.

Applicant respectfully requests reconsideration of the lack of unity objection as between Groups I and IV. In view of Applicant's election of Group I, it is respectfully requested that the claims of Groups I and IV, i.e., claims 1, 3, 4, 11-16 and 23-36, be examined in the instant application.

See also, the sixth paragraph of MPEP 1893.93(d):

> A process is "specially adapted" for the manufacture of a product if the claimed process inherently produces the claimed product with the technical relationship being present between the claimed process and the claimed product. The expression "specially adapted" does not imply that the product could not also be manufactured by a different process.

Lack of Novelty of Group I

While appearing to agree with the above, the Office Action alleges that the claims of Groups I and IV lack unity of invention because the claims of Group I allegedly are not novel. Applicant respectfully disagrees.

The Office Action, mailed February 26, 2001, urges that the Objection is based on the allegation that synthesis of combinatorial libraries of compounds using solid supports and blocking groups is well known in the art. As stated in the Office Action:

> The traversal is on the ground(s) that the special technical feature of group I is not taught by the prior art. This is not found persuasive. Applicants point out that Carell et al. do not teach combinatorial synthesis based on immobilized molecules. Applicants arguments have been considered. However, it is well known in the art to synthesize combinatorial library of compounds using solid supports and blocking groups. Thus, the inventions lack unity.

The Office Action does not dispute Applicant's argument that Carell et al., cited previously by the Office, does not anticipate Group I, and therefore does not destroy the unity of invention as between Groups I and IV. The Office Action alleges that the subject matter of the claims of Group I is well known in the art and therefore lacks novelty. Applicant respectfully disagrees. The Office Action fails to cite any art relating to the novelty of the claims of Group I. The Office Action improperly relies on a bare allegation to allege lack of novelty of the claims of Group I.

Th Office Action improperly relies on a bare allegation

The Office Action provides no support for the bare allegation that "it is well known in the art to synthesize combinatorial library of compounds using solid supports and blocking groups." As stated in MPEP 2144.03, citing in re Ahlert, 424 F.2d 1088, 1091, 165 USPQ 418,420-421 (CCPA 1970), "[A]ssertions of technical facts in areas of esoteric technology must always be supported by citation of some reference work" and "allegations concerning specific 'knowledge' of the prior art, which might be peculiar to a particular art should also be supported." No support for the allegation that "it is well known in the art to synthesize combinatorial library of compounds using solid supports and blocking groups" is provided. Applicant respectfully requests that the Examiner provide support for the allegation. If no support is provided, then th it is respectfully requested that the allegation, and the Objection, be withdrawn.

The claims of Group I are novel over the bare allegation of the Office Action

Moreover, even if the bare allegation of the Office Action is correct, which Applicant does not admit, such allegation does not anticipate the claims of instant Group I and thus does not destroy unity of invention as between Groups I and IV. The claims of Group I are directed to a process for generating a combinatorial set of molecules of core structure M by:

(a) preparing a plurality of immobilized molecules of core structure M, wherein said molecules contain a plurality of reactive moieties, each reactive moiety being blocked by a blocking group, wherein at least three of the blocking groups are independently removable under at least three different conditions, and

(b) removing certain blocking groups and derivatizing the resulting reactive moieties in a preprogrammed, regioselective manner, wherein each member of a combinatorial set is uniquely derivatized at at least one reactive moiety with a unique substituent, thereby generating a combinatorial set of molecules of core structure M.

It is respectfully submitted that the bare allegation of the Office Action does not anticipate this claim. Instant claim 1 requires use of three blocking groups that are independently removable under at least three different conditions in a preprogrammed, regioselective manner. Furthermore, instant claim 1 requires that each member of the combinatorial set produced by the claimed process be uniquely derivatized at at least one reactive moiety with a unique substituent. The bare allegation of the Office Action that "it is well known in the art to synthesize combinatorial library of compounds using solid supports and blocking groups" does not recite these limitations. Therefore, the bare allegation of the Office Action, if accepted as true, does not anticipate the claims of instant Group I, and also does not destroy the unity of invention as between Groups I and IV.

Applicant respectfully petitions for removal of the lack of unity objection as between Groups I and IV, and that these Groups be combined for examination in the instant application.

The finality of the Objection is premature

Furthermore, irrespective of whether the arguments presented in the Office Action are correct or not, the finality of the Objection is premature. The Office Action alleges for the first time that "it is well known in the art to synthesize combinatorial library of compounds using solid supports and blocking groups." Thus, the Office Action has provided a new basis for the allegation of lack of unity of invention while simultaneously making the Objection final. Applicant has not been provided an opportunity to respond to the new basis for the Objection. Therefore, it is respectfully submitted that the finality of the Objection is premature.

Summary

Applicant respectfully requests reconsideration and removal of the lack of unity objection as between Groups I and IV. It is respectfully submitted that the Objection as between Groups I and IV is improper because the Groups are

related as a product and a process specially adapted for preparation of the product. Such related Groups do not lack unity of invention within the meaning of PCT Rule 13.1.

Furthermore, the bare assertion of the Office Action does not anticipate the claims of instant Group I, and, therefore, does not destroy the unity of invention as between Groups I and IV. Also, the finality of the Objection is premature because Applicant has not had the opportunity to respond to the new basis for the Objection set forth in the Office Action.

As noted above, applicant has also submitted a Petition pursuant to 37 C.F.R. §1.144 that was timely filed within 2 months from the mailing of the Office Action requesting reconsideration and removal of the finality of the Lack of Unity Objection.

REQUIREMENT FOR ELECTION OF SPECIES

Applicant respectfully requests removal of the Requirement for Election f Species. It is respectfully submitted that, as described in detail below, the species are so linked as to form a single general inventive concept within the meaning of PCT Rule 13.1 (see, MPEP 1893.03(d)). It is noted that nothing herein should be construed as an admission that the various species present in the instant claims are obvious variants of each other. Furthermore, it is respectfully submitted that the requirement for election of a single species is inconsistent with the subject matter of the instant claims.

Unity of Invention

This application is the U.S. national stage of International Patent Application No. PCT/US97/06509. Therefore, PCT regulations with respect to unity of invention are applicable herein (see, PCT Rule 13, 37 CFR §1.475, and MPEP 1893.03(d)). Unity of invention is based on the basic principle that an applicant has the right to include in a single application those inventions (e.g., species) which are so linked as to form a single general inventive concept (MPEP 1893.03(d)). Applicant respectfully submits that, given the subject

matter of the instant claims, the building blocks (e.g., nucleosides), reactive groups, and protecting groups are so linked as to form a single general inventive concept. Therefore, a requirement to elect a single species in each of these elements is inappropriate.

The instant claims are directed to a combinatorial protecting group strategy for multifunctional molecules. In particular, the claims are directed to products and processes for oligonucleotide synthesis and for preparation of combinatorial libraries. Restriction of the claims to the single species required in the Office Action would negate a fundamental aspect of the subject matter of the instant claims (i.e., the combinatorial aspect).

Restriction of Monomers

The instant claims are directed to products and processes for preparation of the products, where the products are a combinatorial set of molecules of core structure M, or a composition comprising an oligomer. In particular, the claims are directed to (1) oligomers comprising not only of one moiety, but in the case of oligonucleotides, of at least all four of the natural building blocks, and (2) within the oligomer, using different protecting groups at different positions within the oligomer chain (even at the same type of building block).

As described in detail in the application, such products are useful as drugs. Modified oligonucleotides are used in an antisense/triplex DNA approach. Modifications are necessary to provide oligonucleotides of desired cell and nuclear transport properties, while retaining the desired activity. The instant processes provide such various products in a preprogrammed, specific manner requiring only one oligonucleotide synthesis run. In order to fully utilize the products and processes of the instant claims, use of various monomers is required.

Therefore, restriction of the claims to only a single building block, particularly where the products are oligonucleotides, (e.g., a single disclosed species from claim 10; a single disclosed species representing B or B^{R2A} or B^{R2B};

or a single disclosed species from claim 31) is not appropriate sinc these building blocks are all part of a single general inventive concept. The claims are directed to compositions and combinatorial libraries containing oligonucleotides, and processes for preparation of same. If Applicant were required to elect a single disclosed nucleoside species as required, then the search, and any resulting allowed claims, would be limited to oligonucleotides containing only the single elected nucleoside (i.e., TTTTTTT..., GGGGGG..., AAAAAA..., CCCCCCC..., etc.). Such a requirement is inconsistent with the subject matter of the instant claims, and violates Applicant's right to have all species that form a single general inventive concept included in a single application.

Restriction of Reactive Groups and Protecting Groups

Furthermore, the application is directed to a combinatorial protecting group strategy for multifunctional molecules. Restriction to a single reactive group or a single protecting group is inconsistent with the subject matter of the instant claims.

Restriction of the claims to only a single reactive group, e.g., a reactive group of claims 11 or 24, is not appropriate because the claims are directed to products and processes that have different protecting groups at different positions (i.e., on different reactive groups) within the product. To restrict the reactive groups to a single reactive group would negate a fundamental aspect of the instant claims. The different reactive groups are part of the single general inventive concept of the instant claims.

Restriction of the claims to only a single protecting group, e.g., a single R^{4A} or R^{4B} group; or a single R^{2A} or R^{2B} group; or a single group for R^3; is also not appropriate because the claims are directed to different protecting groups at different positions within the product. To restrict the reactive groups to a single reactive group would negate a fundamental aspect of the instant claims. The different protecting groups are part of the single general inventive concept of the instant claims.

Therefore, requiring election of a single disclosed reactiv group or protecting group is inconsistent with the subject matter of the instant claims. Moreover, such requirement violates Applicant's right to have all species that form a single general inventive concept included in a single application (see, MPEP 1893.03(d)).

Summary

For the reasons given above, Applicant respectfully requests removal of the lack of unity objection as between Groups I and IV. Furthermore, Applicant respectfully requests removal of the lack of unity objection as relating to the requirement for election of a single species.

Applicant has also submitted a Petition pursuant to 37 C.F.R. §1.144 that was timely filed within 2 months from the mailing of the Office Action requesting reconsideration and removal of the finality of the Lack of Unity Objection and the Requirement for Election of Species.

DRAWINGS

The Office Action notes that the application was filed with informal drawings. Formal drawings will be submitted at the appropriate time. Any necessary amendment to the specification will be made at that time.

OBJECTION TO THE SPECIFICATION

The Office Action objects to the specification for allegedly failing to provide antecedent basis for the types of solid supports claimed in claim 14. Applicant has amended the specification herein to provide basis for the supports of claim 14. Therefore, it is respectfully submitted that this objection has been overcome.

TRADEMARKS

The Office Action notes that certain trademarks appear in the application and requires that such be properly capitalized and accompanied by generic terminology. Applicant has herein amended the specification to properly identify the registered trademarks WATERS NOVA-PAK C18®, MILLENIUM®,

TEFLON®, SEPHAROSE and SEPHADEX®, and to provide generic terminology for these marks.

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §101

Claims 1, 3, 4 and 11-16 are rejected under 35 U.S.C. §101 as allegedly not being supported by either a specific utility or a well established utility. It is alleged that the combinatorial set of compounds prepared by the claimed methods are themselves the subject of research, and therefore lack utility. Applicant respectfully traverses this rejection.

Relevant Law

The Office has published guidelines for Examiners to use in determining when reviewing patent application for compliance with the "useful invention" (*i.e.*, "utility") requirement of 35 U.S.C. §§ 101 and 112, first paragraph:

(a) Determine what the applicant has claimed, noting any specific embodiments of the invention.

(b) Ensure that the claims define statutory subject matter (i.e., a process, machine, manufacture, composition of matter, or improvement thereof).

(c) If at any time during the examination, it becomes readily apparent that the claimed invention has a well-established utility, do not impose a rejection based on lack of utility. An invention has a well-established utility (1) if a person of ordinary skill in the art would immediately appreciate why the invention is useful based on the characteristics of the invention (*e.g.*, properties or applications of a product or process), and (2) the utility is specific, substantial, and credible.

Examiners are also to review the claims and the supporting written description to determine if the applicant has asserted for the claimed invention any specific and substantial utility that is credible. If the applicant has asserted that the claimed invention is useful for any particular practical purpose (*i.e.*, it has a ''specific and substantial utility') and the assertion would be considered

credible by a person of ordinary skill in the art, then a rejection based on lack of utility may not be imposed.

Credibility is assessed from the perspective of one of ordinary skill in th art in view of the disclosure and any other evidence of record (*e.g.*, test data, affidavits or declarations from experts in the art, patents or printed publications) that is probative of the applicant's assertions. An applicant need only provide one credible assertion of specific and substantial utility for each claimed invention to satisfy the utility requirement.

The instant claims

Instant claim 4, as amended herein, is directed to a process for generating a combinatorial library by:

(a) preparing a plurality of immobilized molecules selected from a nucleoside and a nucleotide; wherein each molecule contains 3 to 10 reactive moieties, each reactive molety being blocked by a blocking group, wherein at least three of the blocking groups on each immobilized molecule are independently removable under at least three different conditions; and

(b) removing each blocking group and derivatizing the resulting reactive moiety in a preprogrammed, regioselective manner; wherein each member of the plurality of immobilized molecules is uniquely derivatized at at least one reactive moiety with a unique substituent, thereby generating a combinatorial library.

Claim 11 further defines the reactive moieties. Claims 12-14 specify that the immobilized molecule is immobilized on a solid support, and specify the solid support. Claims 15 and 16 further define the linker used to immobilized the molecule on the solid support.

Utility of the claimed subject matter

The Office Action alleges that instant claims 1, 3, 4 and 11-26 are not supported by either a specific asserted utility or a well established utility. Applicant respectfully disagrees.

Specifi asserted utility

It is alleged in the Office Action that the specification discloses that the compounds (oligonucleotides) are useful as drugs, which is not specific. In fact, the specification discloses that the compounds prepared by the claimed methods are useful in antisense and triplex DNA therapy (see, *e.g.*, the specification at page 2, lines 15-16 and page 14, lines 14-19).

The Office Action alleges that the specification dos not identify the compounds which would be useful as drugs. It is respectfully submitted that such identification is not necessary. One of ordinary skill in the art would be able to determine, using standard assays known to those of skill in the art, which compounds of a library prepared by the instantly claimed methods would have the requisite biological activity. The biological activity required is a function of the disease, disorder or symptom to be treated. Thus, one of ordinary skill in the art would be able to readily determine, for a given disease, disorder or symptom, which compounds of a given library possess the required biological activity for that disease, disorder or symptom.

It is further alleged that there "is no basis in the specification upon which to conclude that *any* of the compounds encompassed by the library are, or will turn out to be, biologically active after testing," and that the "nucleic acid library prepared by the claimed method would require further research to identify useful oligonucleotides." It is respectfully submitted that the claimed method could be used to prepare libraries containing, in addition to new compounds, known antisense/triplex DNA agents. Therefore, these libraries would contain compounds which are biologically active. No further research would be required to determine that these libraries contained identifiable useful oligonucleotides. Furthermore, as noted above, one of skill in the art would be able to readily determine, using standard assays, which compounds possess requisite activity as antisense agents or for formation of triplex DNA, *e.g.*, for treatment of a given disease, disorder or symptom.

Moreover, the Office Action has not s t forth any reasons for doubting the asserted utility. It was well known in the art, at the time the application was filed, that oligonucleotide analogs are useful as antisense and triplex DNA agents. The instantly claimed methods provide methods for the preparation of such compounds. The instantly claimed methods are applicable to the preparation of a wide variety of oligonucleotide analogs, including known antisense and triplex DNA agents. Given this applicability of the methods, there is no reason for the Office to doubt the asserted specific utility.

Well established utility

It is further alleged in the Office Action that the combinatorial libraries produced by the instantly claimed methods are not supported by a well established utility. Applicant respectfully disagrees.

As noted in the Office Action, "a well established utility is a specific utility which is well known, immediately apparent and implied by the specification based on the disclosure of the properties of a material, alone or taken with the knowledge of one skilled in the art." As described in detail above, the combinatorial libraries prepared by the instantly claimed methods contain, *e.g.*, oligonucleotide analogs. It was well known at the time the application was filed that oligonucleotide analogs have utility as antisense or triplex DNA agents. See, *e.g.*, Uhlmann *et al.* (1990) *Chem. Rev. 90*:543-584 and Beaucage *et al.* (1993) *Tetrahedron 49*:6123-6194, cited in the application (copies enclosed).

Moreover, the libraries produced by the instantly claim process have utility in screening for compounds that are useful as antisense agents. Such libraries contain oligonucleotides and/or oligonucleotide analogs. These libraries were well known to those of skill in the art at the time the application was filed to have utility in screening for antisense agents.

It is further alleged in the application that "the compounds of the claimed libraries are not recognizable as analogous to compounds with a recognized

pharmacol gical (or other) activity." Applicant notes that instant claims 4 and 11-16 are directed to processes for generating combinatorial libraries, not to combinatorial libraries themselves. Moreover, the compounds of the libraries produced by the instantly claimed methods are, *e.g.*, oligonucleotide analogs. As noted above, such compounds are well known to possess activity as antisense or triplex DNA therapeutic agents. Furthermore, the instantly claimed processes may be used to prepare known oligonucleotide analogs, among other compounds. Therefore, contrary to the assertion of the Office Action, data as to the activity of the compounds of the libraries produced by the instantly claimed methods is not required to establish the utility of instant claims 4 and 11-16 because some of the compounds that may be prepared by the methods are known to have antisense and triplex DNA activity.

Applicant respectfully requests reconsideration and removal of this rejection.

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §112, FIRST PARAGRAPH, FOR LACK OF ENABLEMENT

It is alleged in the Office Action that "since the claimed invention is not supported by either a specific and substantial asserted utility or a well established utility, one skilled in the art would not know how to use the claimed invention." Applicant respectfully disagrees.

As described in detail above, the combinatorial libraries prepared by the instantly claimed methods are supported by both a specific utility and a well established utility. Therefore, one of skill in the art would know how to use the instantly claimed methods.

Applicant respectfully requests reconsideration and removal of this rejection.

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §112, FIRST PARAGRAPH, FOR LACK OF WRITTEN DESCRIPTION

Claims 1, 3, 4 and 11-16 are rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. It is alleged that the instant claims do not recite the core structure M of the compounds or other identifying characteristics. While not agreeing with the propriety of this rejection, Applicant has cancelled claims 1 and 3 herein without prejudice or disclaimer. Claims 4 and 11-16 do not recite that the compounds have core structure M. Claim 4 recites that the immobilized compounds are selected from a nucleoside and a nucleotide. The Office Action notes that the specification is directed to oligonucleotide combinatorial libraries. Thus, it is respectfully submitted that the specification does provide adequate written description of the claims, as amended herein.

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §112, FIRST PARAGRAPH, FOR LACK OF ENABLEMENT

Claims 1, 3, 4 and 11-16 are rejected under 35 U.S.C. §112, first paragraph, as allegedly based on a specification which is not enabling. It is urged that, while being enabling for methods of oligonucleotide synthesis, the specification does not provide enablement for synthesis of any other kind of compounds such as oligosaccharides, lipids, vitamins, hormones, peptides or any other drug compounds. While not agreeing with this rejection, Applicant has cancelled claims 1 and 3 herein without prejudice or disclaimer. It is noted that claim 4 has been rewritten as an independent claim incorporating the limitations of the base claim. Claim 4 has also been amended herein to recited that the immobilized molecule is a nucleotide or nucleoside solely in the interest of advancing the prosecution of this application to allowance. Applicant reserves the right to file divisional applications directed to the cancelled subject

matter. In view of these amendments and the above remarks, reconsideration and removal of this rejection is respectfully requested.

REJECTION OF CLAIM 16 UNDER 35 U.S.C. §112, FIRST PARAGRAPH, FOR LACK OF WRITTEN DESCRIPTION

Claim 16 is rejected under 35 U.S.C. §112, first paragraph, as allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. It is alleged that the specification does not provide an adequate representation regarding the allegedly open ended "derivatives thereof." While not agreeing with the propriety of this rejection, applicant has deleted this recitation from claim 16. This amendment to claim 16 is presented solely in th interest of advancing the prosecution of this application to allowance. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 1, 3, 4 and 11-16 are rejected under 35 U.S.C. §112, second paragraph, as allegedly failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicant requests reconsideration of this rejection in view of the amendments to the claims herein and the following remarks. Applicant notes that claim 1 has been cancelled herein without prejudice or disclaimer. Claim 4 has been rewritten as an independent claim incorporating the limitations of claim 1. The amendments to the claims herein are made solely to advance the prosecution of this application to allowance and are not made to avoid any art of record. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

"A combinatorial s t of molecules of core structure M"

Claim 1 is rejected as allegedly being indefinite for reciting "a combinatorial set of molecules of core structure M." In particular, clarification is requested as to the meaning of "combinatorial set of molecules." The recitation "combinatorial set of molecules" has been amended in claim 4 to recite "combinatorial library." A "combinatorial library," in the context of instant claim 4, is any set of molecules that may be prepared by the claimed process.

"Preparing a plurality of immobilized compounds of core structure M"

Claim 1 is rejected as allegedly being indefinite for reciting "preparing a plurality of immobilized compounds of core structure M." Clarification is requested as to how the compounds are prepared which are immobilized. Claim 4, as amended, recites a plurality of immobilized compounds molecules selected from a nucleoside and a nucleotide. The specification provides in the Examples methods for immobilizing such compounds. Reconsideration and removal of this rejection is respectfully requested.

"Said molecules contain a plurality of reactive moieties"

Claim 1 is rejected for reciting "said molecules contain a plurality of reactive moieties." Clarification is requested regarding what is meant by molecules contain a plurality of reactive moieties. Claim 4, as amended, recites "wherein each molecule contains 3 to 10 reactive moieties." It is respectfully submitted that this recitation makes clear that each molecule has 3 to 10 reactive moieties.

"At least three of the blocking groups..."

Claim 1 is rejected for reciting "at least three of the blocking groups are independently removable under at least three different conditions." Clarification is requested as to what is meant by at least three blocking groups. Claim 4, as amended, recites "at least three of the blocking groups on each immobilized molecule are independently removable under at least three different conditions."

It is respectfully submitted that this recitation makes clear that the at least three blocking groups are on the same compound.

Clarification is also requested as to what is meant by at least three different conditions. As described in the specification (see, *e.g.*, page 5, lines 14-16), the "at least three different conditions" refer to conditions that selectively remove one of the blocking groups without removing the others (principle of orthogonality).

"Removing certain blocking groups"

Claim 1 is rejected for reciting "removing certain blocking groups." Clarification is requested as to what applicant means by certain blocking groups. As amended, claim 4 recites "removing each blocking group." These blocking groups are present on one molecule and are each selectively removed for derivatization of the molecule.

"Preprogrammed, regioselective manner"

Claim 1 is rejected for reciting "preprogrammed, regioselective manner." Clarification is requested as to the meaning of "preprogrammed" and "regioselective." "Preprogrammed" refers to the fact that each blocking group may be removed according to known conditions. Thus, one of skill in the art can predict, based on the library prepared, how to remove each blocking group selectively. Automatic or robotic machines may be employed for this purpose, but are not required. "Regioselective" is to be given its usual meaning in the art. Thus, each blocking group at a given position on the molecule may be removed selectively over other blocking groups in other positions of the molecule.

"The immobilized molecule"

Claim 3 is rejected for reciting "the immobilized molecule." It is alleged that there is insufficient antecedent basis for this recitation. While not necessarily agreeing with this rejection, applicant has cancelled claim 3 herein

without prejudice or disclaimer. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

"Small molecule drug compound"

Claim 4 is rejected for reciting "small molecule drug compound." Clarification is requested. While not necessarily agreeing with this rejection, claim 4 has been amended herein to delete this recitation without prejudice or disclaimer. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

Claims 11 and 12

Claims 11 and 12 are rejected for being dependent on non-elected claim 2. Claims 11 and 12 are amended herein to be dependent on claim 4. Claim 2 is cancelled herein without prejudice or disclaimer. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

"The molecule"

Claim 12 is rejected for reciting "the molecule." Claim 12 has been amended herein to recite "the immobilized molecules." It is respectfully submitted that this recitation has antecedent basis in base claim 4.

"The bottom of a microtiter"

Claim 13 is rejected for reciting "the bottom of a microtiter." Claim 13, as amended herein, recited "bottom surface of a microtiter plate." It is respectfully submitted that antecedent basis for this recitation is not required.

Markush format

Claim 13 is rejected as allegedly reciting an improper Markush group. Applicant has amended this claim as suggested by the Examiner in the interest of advancing the prosecution of this application to allowance.

Trademarks/trade names

Claim 14 is rejected for reciting trademarks and/or trade names. Applicant has amended claim 14 herein to replace these recitations with generic terminology. No new matter has been added.

"Can be"

Claim 15 is rejected for reciting "can be" cleaved. Claim 15, as amended herein, recites "is cleavable." It is respectfully submitted that this recitation is not indefinite.

"Derivatives thereof"

Claim 16 is rejected for reciting "derivatives thereof." Applicant has amended claim 16 herein to delete this recitation solely in the interest of advancing the prosecution of this application to allowance. Applicant reserves the right to file divisional applications directed to any cancelled subject matter.

REJECTION OF CLAIMS 1, 3 AND 11 UNDER 35 U.S.C. §102(b) OVER MONTAL *et al.*

Claims 1, 3 and 11 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by the disclosure of Montal *et al.* (1990) *PNAS(USA) 87(18)*:6929-6933. Applicant respectfully traverses this rejection.

Relevant Law

Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration. In re Spada, 15 USPQ2d 1655 (Fed. Cir, 1990), In re Bond, 15 USPQ 1566 (Fed. Cir. 1990), Soundscriber Corp. v. U.S., 360 F.2d 954, 148 USPQ 298, 301, adopted 149 USPQ 640 (Ct. Cl.) 1966. See, also, Richardson v. Suzuki Motor Co., 868 F.2d 1226,1236, 9 USPQ2d 1913,1920 (Fed. Cir.), cert. denied, 110 S.Ct. 154 (1989). "[A]ll limitations in the claims must be found in the reference, since the claims measure the invention". In re Lang, 644 F.2d 856, 862, 209 USPQ 288, 293 (CCPA 1981). Moreover it is incumbent on Examiner to identify wherein each and every facet of the claimed invention is disclosed in the reference. Lindemann Maschinen-fabrik Gmbh v. American Hoist and Derrick Co., 730 F.2d 1452, 221 USPQ 481 (Fed. Cir. 1984). Further, the reference must describe the invention as claimed sufficiently to have placed a person of ordinary skill in the art in possession of the invention.

Th instant claims

The instant claims are described above.

Differences between the disclosure of Montal *et al.* and the instant claims

Montal *et al.* discloses the 9-amino acid template K*KK*PGK*EK*G, where K* refers to N^{ϵ}-9-fluorenylmethoxycarbonyllysine, as a template for peptide synthesis. This template does not contain at least three blocking groups independently removable under at least three different conditions, as required by the instant claims. In fact, this template does not contain any blocking groups. As noted above, the instant claims are directed to processes for combinatorial library synthesis using compounds possessing at least three different blocking groups removable under at least three different conditions as a template. Montal *et al.* does not disclose such a process. Therefore, the instant claims are not anticipated by the disclosure of Montal *et al.*

REJECTION OF CLAIMS 1, 3, 4 AND 11-16 UNDER 35 U.S.C. §102(b) OVER SAUL *et al.*

Claims 1, 3, 4 and 11-16 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by the disclosure of Saul *et al.* (International Patent Application Publication No. WO 95/13538). Applicant respectfully traverses this rejection.

Relevant Law

The relevant law is described above.

The instant claims

The instant claims are described above.

Differences between the disclosure of Saul *et al.* and the instant claims

Saul *et al.* discloses a method of producing a chemical library containing a core molecule having 3-10 active functionalities. The cited reference discloses at page 22, line 28 to page 23, line 7 that "protective groups may have been employed" and that one example of an amino protective group is an FMOC group. Saul *et al.* further discloses in this recitation that the protective groups may be removed under standard conditions. The reference does not disclose

that each reactive moiety on the core molecule is blocked by a blocking group, wherein at least three of the blocking groups are independently removable under at least three different conditions, as required by the instant claims. Therefore, Saul *et al.* does not disclose each and every element of the instant claims. Thus, the instant claims are not anticipated by the disclosure of Saul *et al.*

REJECTION OF CLAIMS 1, 3 AND 4 UNDER 35 U.S.C. §102(b) OVER STENGELE *et al.*

Claims 1, 3 and 4 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by the disclosure of Stengele *et al.* (1990) *Tetrahedron Lett. 31(18)*:2549-2552. Applicant respectfully traverses this rejection.

Relevant Law

The relevant law is described above.

The instant claims

The instant claims are described above.

Differences between the disclosure of Stengele *et al.* and the instant claims

Stengele *et al.* discloses synthesis of deoxyoligonucleotides using an NPE/NPEOC strategy. The cited reference discloses solid phase synthesis of deoxyoligonucleotides using dimethoxytrityl, NPE and NPEOC blocking groups. The cited reference discloses that the NPE and NPEOC groups are removed under the same conditions (see, page 1551, lines 4-5 of the cited reference). The reference does not disclose a strategy using at least three blocking groups that are independently removable under at least three different conditions, as required by the instant claims. Therefore, the instant claims are not anticipated by the disclosure of Stengele *et al.*

* * *

In view f the abov , reconsideration and all wance of the application is respectfully requested.

Respectfully submitted,
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Köster *et al.*

Serial No.: 09/171,625

Confirmation No.: 8272

Filed: July 2, 1999

For: A COMBINATORIAL PROTECTING GROUP STRATEGY FOR MULTIFUNCTIONAL MOLECULES

Art Unit: 1627

Examiner: Ponnaluri, P.

I hereby certify that this paper and the attached papers are being deposited with the United States Postal Service as first class mail in an envelope addressed to: Commissioner for Patents Washington, D.C. 20231, on this date.

08/27/01
Date

Rita Jennings
Rita Jennings

MARKED UP PARAGRAPHS AND CLAIMS IN ACCORDANCE WITH 37 C.F.R. §1.121

Please amend the paragraph at page 28, line 16 through page 29, line 23 as follows:

^{1}H (400 and 250 MHz) and ^{13}C)101 and 63 MHz) NMR spectra were recorded on a Bruker AMX 400 and a AC 250-P instrument. Samples were dissolved in the presence of tetramethylsilane as internal standard, unless otherwise stated. ^{31}P NMR spectra were recorded on a Varian Gemini 200 instrument. Internal standard: phosphoric acid in the solvent used for the sample (δ = 0.00 ppm), Chemical shifts are given in ppm. Mass spectra were obtained on a Finnigan MAT 311A mass spectrometer under EI conditions, a VG Analytical 70-250S mass spectrometer under FAB conditions (matrix: 3-nitro-benzyl alcohol, Xenon bombardment) and a Finnigan MAT Vision 2000 mass spectrometer under MALDI-TOF conditions (matrix solution: 0.7 mol/ 13-hydroxy picolinic acid and 0.07 mol/ 1 ammonium citrate in acetonitrile/ water, 1/1, v/v). Elementary analyses were performed by the analytical department of the Institute of Organic Chemistry, University of Hamburg. Thin layer chromatography (tlc) was carried out on 60 PF_{254} silica gel coated alumina sheets (Merck, Darmstadt, No 5562). Trityl and sugar containing compounds are visualized with sugar spray reagent (0.5 ml 4-metoxybenzaldehyde, 9 ml

ethanol, 0.5 ml c ncentrat d sulfuric acid and 0.1 ml glacial acetic acid) by heating with a fan or on a hot plate. p-Nitrophenyl ester containing compounds are visualized by ammonia vapour. Column chromatography was performed using silica gel from Merck. HPLC results were obtained on a Waters chromatography systems 625 LC with a photodiodearray detector 996 and using reversed phase columns ([Waters Nova-Pak C18] WATERS NOVA-PAK C18® (octadecyl silica gel) column, 60 Å, 4 μm particles, 3.9 x 300mm, software: [Millenium] MILLENIUM® 2.0, eluants were: 0.1 M triethylammonium acetate at pH 7.0 (A) and acetonitrile (B); the column was equilibrated at 30°C at 1ml per min, with 95% A/ 5% B, v/v, with elution using a linear gradient from 5% to 40% B in 40 min, monitored at 254 nm). Spectrophotometric measurements in the UV/ Vis region were performed on a Beckman UV35 and a LKB Ultrospec Plus UV/ Vis spectrophotometer. Solvents were dried and purified before use according to standard procedures. Extractions were monitored by tlc to optimize completion of extraction.

Please amend claims 4, 11-16 and 26-28 as follows:

4. (Amended) A process [of claim 3, wherein the low molecular weight compound is] for generating a combinatorial library, comprising the steps of:

(a) preparing a plurality of immobilized molecules selected from [the group consisting of a saccharide, aminosugar, deoxysugar,] a nucleoside[,] and a nucleotide[, coenzyme, amino acid, lipid, steroid, vitamin, hormone, alkaloid and small molecule drug compound]; wherein each molecule contains 3 to 10 reactive moieties, each reactive moiety being blocked by a blocking group, wherein at least three of the blocking groups on each immobilized molecule are independently removable under at least three different conditions; and

(b) removing each blocking group and derivatizing the resulting reactive moiety in a preprogrammed, regioselective manner; wherein each member of the plurality of immobilized molecules is uniquely derivatized at at least one reactive moiety with a unique substituent, thereby generating a combinatorial library.

11. (Amended) A process of claim [1 or 2] 4, wherein the reactive moieties are selected from [the group consisting of] OH, SH, NH_2, CO_2H, SOH, SO_2H, SO_3H, CHO, keto, phosphate, phosphite, phosphoamidite, halogen, CN, CNS, NCS[,] and NCO [and derivatives thereof].

12. (Amended) A process of claim [1 or 2] 4, wherein the [molecule has] immobilized molecules have been immobilized based on linkage to a solid support.

13. (Amended) A process of claim 12, wherein the solid support is selected from beads, flat supports, [wafers with or without pits and/or channels, the bottom of a microtiter plate or] wafers with pits, wafers without pits, wafers with channels, wafers without channels, bottom surface of a microtiter plate, and [the] inner walls of a capillary.

14. (Amended) A process of claim 13, wherein the beads are comprised of a material selected from polystyrene, polyamide, cellulose, [Sephadex, Sepharose] agarose, dextran cross-linked with epichlorohydrin, silica gel, controlled pore glass (CPG), and [teflon] polytetrafluoroethylene.

15. (Amended) A process of claim 12, wherein the linkage [can be cleaved] is cleavable under acidic, alkaline, neutral or photolytic conditions.

16. (Amended) A process of claim 15, wherein the linkage is selected from [the group consisting of] trityl ether, ester, β-benzoylpropionyl, levulinyl, disulfide[,] and sulfenyl [and derivatives thereof].

26. (Amended) A combinatorial [set of compounds according to claim 23] library wherein the compounds comprising the library are selected from [the group consisting of a: saccharide, aminosugar, deoxysugar, nucleoside,

nucleotide, coenzyme, amino acid, lipid, ster id, vitamin, hormone, alkaloid and small molecule drug compound] nucleosides and nucleotides, wherein each compound has from 3 to 10 reactive moieties having blocking groups on the reactive moieties, wherein at least three blocking groups are independently removable under different conditions, thereby allowing selective derivatization after deblocking, and wherein one reactive moiety is utilized for immobilization.

27. (Amended) A combinatorial [set of oligomeric compounds] library according to claim [23] 26, [selected from the group consisting of an oligosaccharide, oligopeptide and oligonucleotide] wherein the compounds of the library are oligonucleotides.

28. (Amended) A combinatorial [set of oligomeric compounds] library of claim [23] 26 in which at one or more positions in the sequence a preselected set of building blocks is incorporated.

Chemical Reviews

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Antisense Oligonucleotides: A New Therapeutic Principle

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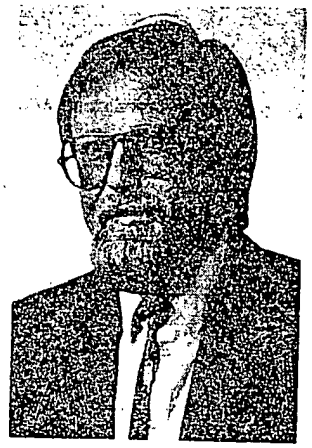
Contents



Eugen Uhlmann was born on March 15, 1953, in Bad Schussenried, Germany. He attended the University of Konstanz, where he obtained his Diplom degree (1978) and Ph.D. degree (1981, with Prof. W. Pfleiderer). After working at Hoechst AG, Frankfurt (1982–1984), he spent 1 year studying with Prof. J. A. Smith at the Massachusetts General Hospital and Harvard Medical School, Boston. In 1986 he joined Hoechst AG, Frankfurt, where is responsible for oligonucleotide synthesis. His research interests are in the synthesis of antiviral nucleosides/nucleotides and oligonucleotides, gene synthesis for the production of recombinant proteins, and protein engineering and structure/function relationship studies.

Anuschirwan Peyman was born on October 19, 1958, in Freiburg i.Br., F.R.G. He attended Albert-Ludwigs Universität Freiburg from 1977 to 1983, where he received his Diplom in Chemistry. His Ph.D. work (1983–1986) was carried out at the Universität Freiburg under the supervision of Prof. Ch. Rüchardt. After a postdoctoral stay (1987–1988) with Prof. J. R. Knowles at Harvard University, he became a member of the Peptide/Nucleoside Group in the Pharmaceutical Division of Hoechst AG, Frankfurt, F.R.G. His research interests are in protein engineering, enzyme mechanism studies, and the synthesis of oligonucleotides and antiviral compounds.

I. Introduction

Even today, new drugs are usually not discovered by rational drug design even though this would be the dream of the medically oriented chemist. On average, it is still necessary to synthesize and test about 10 000 new compounds in order to discover a new active substance worth development. In many cases the active substance is to be directed against proteins such as enzymes, receptors, or ion channels, the structure and mode of action of which are usually very complicated and often incompletely understood. On the other hand, therapeutic intervention at the level of the nucleic acid appears to offer a number of advantages.

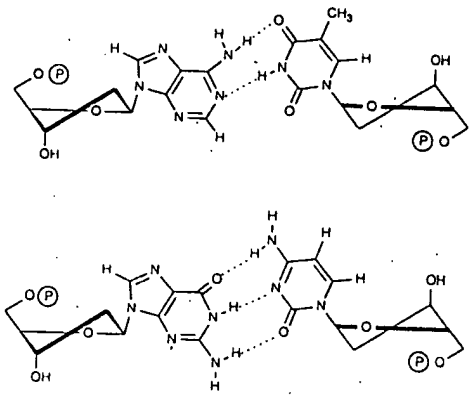


Figure 1. Watson–Crick base pairing.

On transcription, every gene gives rise to a relatively large number of copies of messenger ribonucleic acid (mRNA), which is translated into a large number of protein molecules. This is why inhibition of gene expression ought to be more efficient than inhibition of the resulting protein product. There are already a number of drugs on the market whose activity is based on direct interaction with deoxyribonucleic acid (DNA). Many of these compounds, which are mainly used for chemotherapy, intercalate or bind specifically only to DNA. If the recognition step, the binding to the DNA, is followed by a response, according to Hurley and Boyd[1] the DNA should be regarded as a receptor in the pharmacological sense. Classical drugs such as adriamycin, bleomycin, or cisplatin are, however, unable to exhaust the sequence information contained in the nucleic acids and thus do not act specifically on particular genes. It is, however, possible to achieve such sequence-specific recognition of nucleic acids using synthetic oligonucleotides that bind specifically by hydrogen bonding to complementary nucleic acids. These compounds are called antisense oligonucleotides based on their binding to the target sequence (sense strand). Since, statistically, the base sequence of a 17-mer oligonucleotide occurs just once in the sequence of the human genome, extremely selective intervention ought to be possible with antisense oligonucleotides of this length. An additional point in favor of therapeutic use of oligonucleotides is that they occur endogenously in eucaryotic cells.[2] Moreover, the antisense principle is also used in nature to regulate gene expression. In both procaryotic and eucaryotic cells there is known to be a natural method of regulation based on the binding of complementary nucleotide sequences (antisense RNA) to particular nucleic acids.[3-7] Thus, if the nucleotide sequence of the target molecule is known, it is possible to write down directly the chemical formula of the inhibitor, corresponding to the base sequence of the antisense oligonucleotide, which amounts to rational drug design. Unlike rational drug design when the target molecules are proteins, there is no need for X-ray structure and NMR analyses.

However, it ought to be emphasized that antisense oligonucleotides can be used not only to inhibit but also to activate gene expression. This is possible, for example, indirectly by suppressing the biosynthesis of a natural repressor[4] or directly by reducing termination of transcription.[8]

A. Antisense RNA: A Natural Gen Expression Control System

Gene expression in cells is normally controlled by DNA-binding proteins, repressors, and activators. Only recently have regulatory RNA sequences been established as direct repressors of gene expression. This so-called antisense RNA is produced from the corresponding antisense genes by transcription. In the simplest case this can take place by transcription of the complementary (antisense) DNA strand. The antisense RNA then binds, via Watson–Crick base pairing,[9] to the complementary (sense) target nucleic acid. Transcription of both complementary strands in the same region of the DNA was first observed in 1969 by Bovre and Szybalski on phage lambda.[10] However, at that time it was possible only to speculate about a regulatory role of antisense RNA. Not until 1983 did Simons and Kleckner[11] detect in procaryotes an RNA that was complementary to the ribosome-binding site, including the start codon, of a gene and suppressed its expression in a specific manner. Interestingly, 6 years before, Paterson et al. had already directed complementary single-stranded DNA against mRNA and thus achieved suppression of mRNA translation in a cell-free system.[12] They introduced the term HART (*h*ybrid *a*rrested *t*ranslation) for this. As expected, artificial antisense RNA able to inhibit the expression of any desired gene is widely used in fundamental research. However, it is also being investigated extensively with a view to use in therapy as well as in agriculture, especially in crop protection. Since this antisense RNA technology has been dealt with comprehensively in some review articles,[3-7,13] we will focus on synthetic antisense oligonucleotides.

B. Antisense Oligonucleotides

Zamecnik and Stephenson were the first to propose, in 1978, the use of synthetic antisense oligonucleotides for therapeutic purposes.[14,15] They were able with a 13-mer oligonucleotide that was complementary to the RNA of Rous sarcoma virus to inhibit the growth of this virus in cell culture. The specific inhibition is based on the specific Watson–Crick base pairing (Figure 1) between the heterocyclic bases of the antisense oligonucleotide and of the viral nucleic acid. The process of binding of the oligonucleotides to a complementary nucleic acid is called hybridization.

Various cellular processes can be inhibited depending on where the oligonucleotide hybridizes on single-stranded regions of the DNA or mRNA. A simple model describes the inhibition of protein biosynthesis by an antisense oligonucleotide being bound to the mRNA (Figure 2). Although this illustrative model does not always reflect the actual mechanisms, nevertheless it reveals the essential steps in the new principle and the problems associated with it: for the antisense oligonucleotide to be able to inhibit translation it must reach the interior of the cell unaltered. The requirements for this are, on the one hand, stability of the oligonucleotide toward extra- and intracellular enzymes but also, on the other hand, ability to penetrate through the cell membrane. Once it has reached the cytoplasm it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein. In order to meet all these requirements it is necessary for normal oligonucleotides to be chemically modified in a suitable manner. Hence we describe below the synthesis of normal and of modified oligonucleotides and then discuss the properties of these compounds that make some of them good inhibitors, and some of them less effective inhibitors, of gene expression.

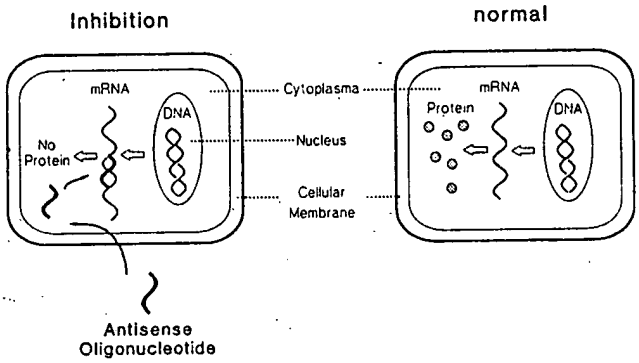


Figure 2. Principle of action of the antisense oligonucleotides.

II. Methods for the Synthesis of Oligonucleotides and Their Analogues

A. Unmodified Oligonucleotides

The preparation of unmodified oligodeoxynucleotides has been the center of interest of many research groups in the past decade, not least because of their use in genetic engineering.[16] The methods and problems of solid-phase and "manual" synthesis have been described in detail in several review articles,[17-27] the most timely written by Sonveaux,[17] so mention will be made here of only the most important points, the chain-extension steps in the conventional methods.

The synthesis via phosphoramidites according to Caruthers,[28] originally introduced by Letsinger[29] as the phosphite triester method, is currently the most efficient method for preparing oligodeoxynucleotides. It entails the 5′-OH group of the growing DNA chain being reacted with a nucleoside 3′-β-cyanoethyl *N*,*N*-diisopropylphosphoramidite with catalysis by 1*H*-tetrazole and the resulting phosphite triester being oxidized immediately with I_2 to the phosphotriester (Figure 3). The coupling yield in the amidite method is >99%,[30] and a synthesis cycle takes about 8 min. It is possible in this way to construct oligomers having up to 175 nucleotides using automatic DNA synthesizers.[31] Mention may be made of the possibility of carrying out 5′-phosphorylation as part of a normal synthesis cycle.[32]

In the past 2 years the H-phosphonate method, which was described for the first time by Todd in 1957,[33] has become reestablished and has in some cases replaced the amidite method, particularly because the synthons are easier to handle and no phosphate protective group is employed.[34-39] This method entails the 5′-OH group of the growing DNA chain being reacted with a nucleoside 3′-H-phosphonate (Figure 4). The condensing agents used are sterically hindered carbonyl chlorides such as adamantoyl or pivaloyl chloride. The resulting phosphite diester is oxidized with *tert*-butyl hydroperoxide or iodine to the phosphotriester only after construction of the chain is complete, in contrast to the amidite method. The H-phosphonate method can also

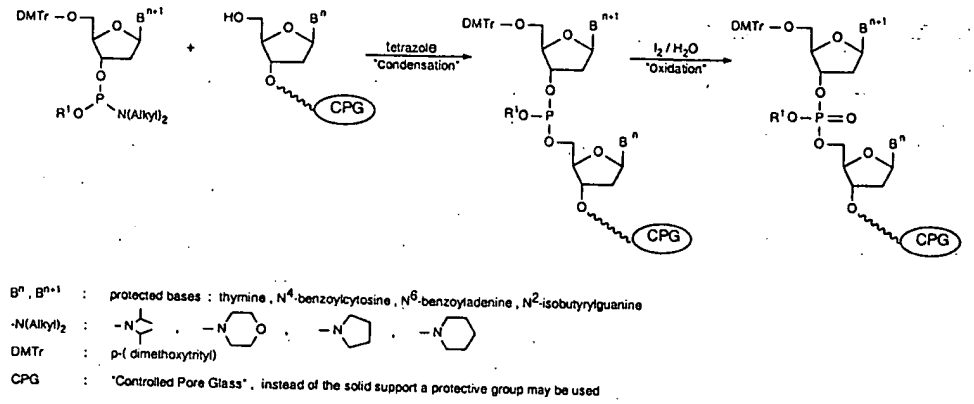


Figure 3. Amidite method.

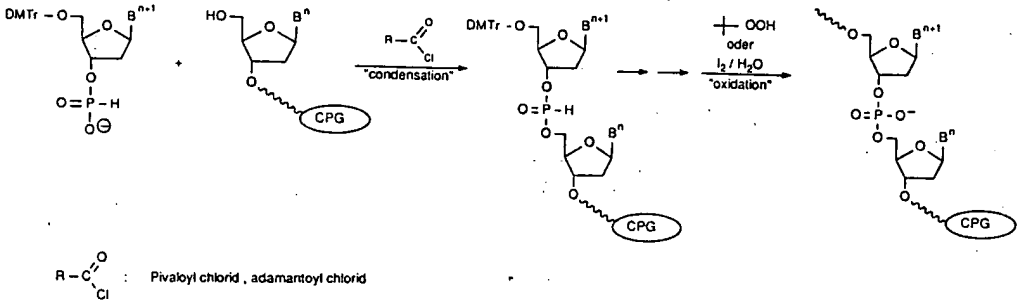


Figure 4. H-Phosphonate method.

be used to prepare oligomers of considerable chain length; e.g., Froehler et al. have reported the synthesis of a 107-mer.[36]

The phosphotriester method,[17,26] which dominated oligodeoxynucleotide preparation for a long time, is still regarded as the method of choice for large-scale synthesis,[26] although the H-phosphonate method has gained ground here, too.[40] In this method the required phosphotriester is obtained in one step from the 5′-OH group of the growing chain and the nucleoside 3′-phosphodiester units (Figure 5). The condensation is brought about with 3-nitro-1,2,4-triazolides of an arenesulfonic acid, preferably 2,4,6-triisopropylbenzenesulfonic acid[41,42] (TIPS) or 8-quinolinesulfonic acid[43,44] (QSNT). A further improvement was achieved by using nucleophilic catalysts, e.g., 4-substituted pyridine *N*-oxides, which, intramolecularly attached to the phosphate protecting group, jack up the coupling yield to 98% and reduce the time taken by a synthesis cycle to 7–8 min.[45]

The synthetic oligodeoxynucleotides are purified by polyacrylamide gel electrophoresis, followed by removal of salts, or by HPLC.[46,47] Capillary gel electrophoresis[48,49] is proving to be extremely useful for the analysis and fractionation of small amounts of oligodeoxynucleotides and may be regarded as the future method of choice. Sequence analysis of purified oligodeoxynucleotides is possible by three methods: The first method is that of Maxam and Gilbert,[50] which is based on chemical cleavage and subsequent gel electrophoresis. The second method is the wandering-spot method,[51] in which the oligodeoxynucleotide is subjected to a partial enzymatic digestion and then fractionated by two-dimensional electrophoresis. A third method has been reported by Ansorge et al. using chemical degradation on oligonucleotides bound to Hybond paper.[52] Sequences of shorter oligodeoxynucleotides can also be analyzed by FAB[53] or by plasma desorption

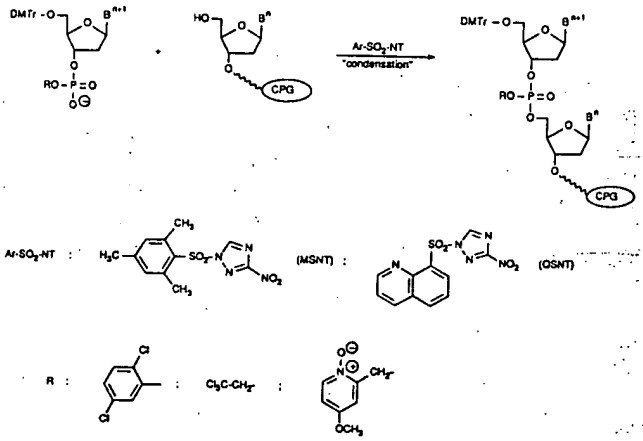


Figure 5. Phosphotriester method.

mass spectroscopy.[54,55] Since these rapid and accurate analytical methods can also be applied to modified compounds,[56] they could well achieve greater importance.

When "normal" oligodeoxynucleotides were used as antisense oligonucleotides, the problems described in section III emerged—i.e., instability to nucleases and insufficient membrane penetration. This and, of course, purely academic interest gave rise to a variety of modifications (Figure 6), which will be dealt with below.

B. Oligodeoxynucleotides with a Modified Internucleotide Phosphat Residue

1. Methylphosphonates

In order to improve uptake by cells and extend the biological half-life, Miller and Ts'O have concentrated on eliminating the negative charge on the internucleotide phosphate bridge.[57–59] In the methylphosphonate oligodeoxynucleotides, which they called

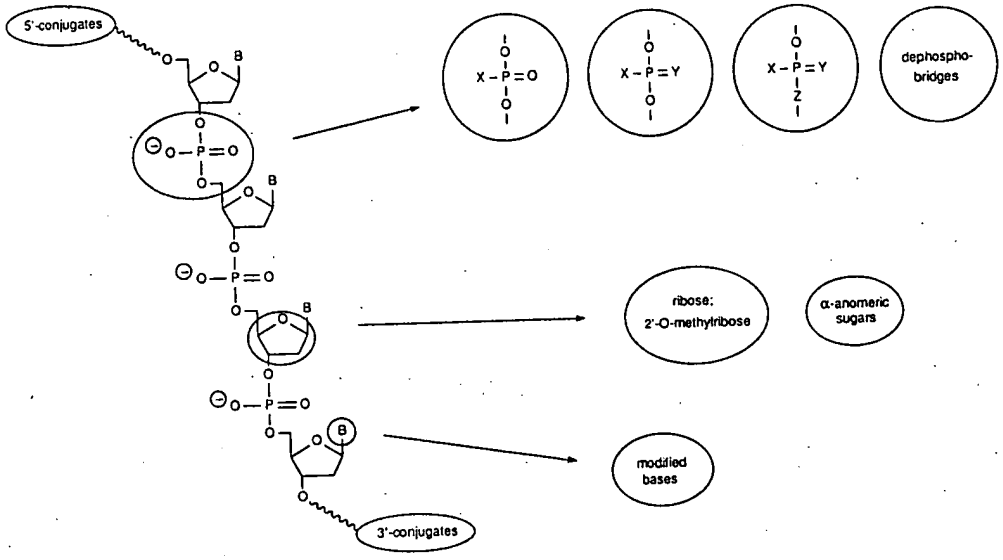


Figure 6. Possibilities for modifying oligonucleotides.

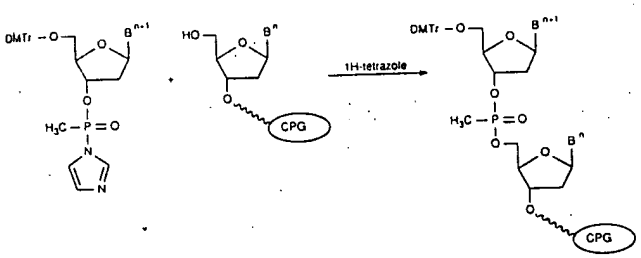


Figure 7. Preparation of oligonucleotide methylphosphonates by the phosphotriester method.

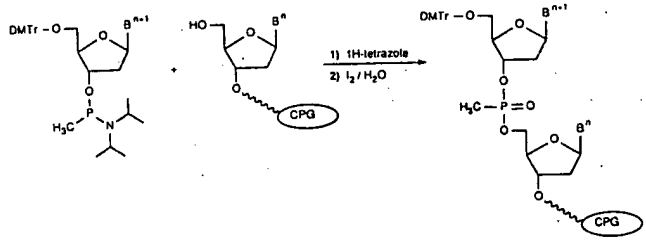


Figure 8. Preparation of oligonucleotide methylphosphonates by the amidite method.

MATAGENES (*ma*sking *ta*pe for *g*ene *e*xpression),[59] the negatively charged phosphate oxygen is replaced by a methyl group, which is neutral and sterically undemanding.

The methylphosphonate oligodeoxynucleotides can be obtained in two ways whose chemistry is based on that of the "natural" representatives although the condensation reactions take place very much more slowly than with normal phosphates. The triester method was used first to condense triethylammonium salts of nucleoside 3′-methylphosphonates with 5′-unprotected nucleosides or oligomers using coupling reagents such as 1-(2-mesitylsulfonyl)-3-nitro-1,2,4-triazole (MSNT).[60,61] As an alternative, Agarwal[62] used methylphosphonic acid bis(1,2,4-triazolides), which, additionally activated with benzenesulfonic acid tetrazolides or 1*H*-tetrazole,[63] are employed for the condensation. This procedure, which is based on methylphosphonic dichloridite[64] as combined phosponylating and condensing reagent, derived from the strategy formerly used in the triester method[21] and was eventually optimized by Miller and Ts'O[65] by using 5′-protected nucleoside 3′-methylphosphonic acid imidazolides (Figure 7). The yields in each coupling step are 88–92%, which allows 15-mers to be synthesized in an isolated yield of 4%. In this connection the approach of van Boom et al.[66] should be mentioned in which the methylphosphonate oligodeoxynucleotides were prepared via bis-*O,O*-(1-benzotriazolyl) methylphosphonates.

However, a more efficient synthesis is via phosphonoamidites, which were described for the first time by Jäger and Engels[67] after they had previously introduced methylphosphonous dichloridite as central synthon,[68] which was used later on by Köster et al.[69] for the solid-phase synthesis of methylphosphonate oligodeoxynucleotides. However, whereas Jäger and Engels used collidine hydrochloride as activator and obtained only moderate yields (about 80%), Dorman et al.[63] achieved yields of over 90% with imidazole. The synthesis of methylphosphonate oligodeoxynucleotides using methylphosphonoamidites on a solid support[70,71] takes place with yields of 96–97% in each coupling step[71] and may be regarded as the method of choice (Figure 8).

The use of these synthons also allows unmodified oligodeoxynucleotides with only one or with several methylphosphonate bridges at any desired point in the molecule to be synthesized using the usual synthesis cycle.[70] This is of particular interest for the studies of stability to nucleases discussed in section III.D.

Several authors have described the synthesis of methylphosphonate oligodeoxynucleotides via an Arbusov reaction with dinucleoside *O*-methyl phosphites and methyl iodide,[72-75] but this has not proven useful, inter alia, because of the low yields of maximum 70%.[75]

Since the internucleotide methylphosphonate bridge is more base labile than the natural internucleotide linkage,[69,76] milder conditions are necessary for cleavage from the support and deprotection. Whereas the latter is normally carried out with concentrated NH_4OH at 60 °C for 8 h, in the case of the methylphosphonate it is carried out at room temperature for only 2 h and then with ethylenediamine/ethanol (1:1) for 7 h, likewise at room temperature.[65,70] The use of only ethylenediamine/ethanol or *tert*-butylamine in methanol at 0 °C has also been proposed.[63]

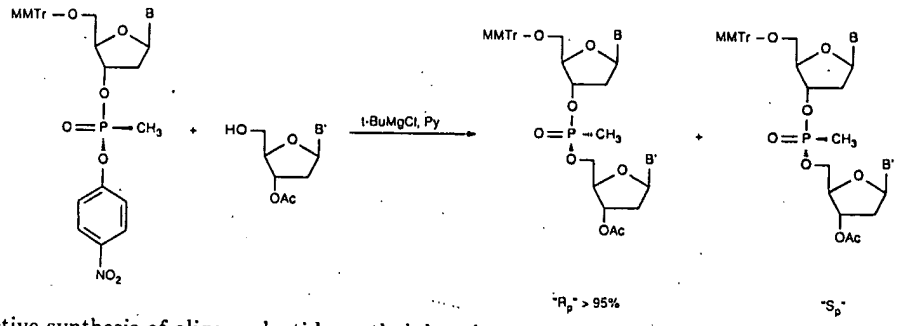


Figure 9. Stereoselective synthesis of oligonucleotide methylphosphonates.

Another problem is posed by the chirality of the methylphosphonate bridge, which can have the R_p or S_p configuration (Figure 9). Attempts were made early on to separate diastereomeric dinucleotides by chromatographic methods[60] and to achieve a configurational assignment. As Katti and Agarwal showed,[77] separation is more effective when chiral protective groups are used, such as 1-menthoxy carbonate in the 3′ position. The configuration of the S_p diastereomer of $dA_{Me}T$ was elucidated by X-ray structural analysis,[78] and the diastereomers of $d(A_{Me}A)$ were individually assigned by ^1H NMR NOE experiments.[79] Similar investigations have recently been undertaken by Engels on $T_{Me}T$.[80] The ^{13}C NMR signals for one diastereomer of $T_{Me}T$ were assigned.[74] None of the syntheses described above takes place selectively. This means that an *n*-meric methylphosphonate oligodeoxynucleotide is obtained as a mixture of 2^n diastereomers, which is an unsatisfactory result for many investigations. A first promising approach to stereoselective synthesis has been introduced by Stec et al.[81,82] This entails the R_p and S_p diastereomers of $T_{Me}T$ being obtained by stereospecific reaction of the P-chiral nucleotide components 5′-*O*-(monomethoxytrityl)thymidine 3′-*O*-(*O*-(4-nitrophenylmethanephosphonate) and 3′-*O*-acetylthymidine (Figure 9). The 5′-OH group is activated by *tert*-butylmagnesium chloride, and the reaction takes place with inversion on the phosphorus. The stereospecificity is >95%, but the chemical yield of ca. 70% means that there is little hope of successful use in solid-phase synthesis. Tetramers with all-R_p or all-S_p configuration are obtained by stepwise reaction. Engels is currently attempting to achieve diastereoselectivity by using chiral amidites, with proline in place of the diisopropylamino group.[83]

The methyl group can be replaced by other alkyl or by aryl moieties. Thus, phenylphosphonate[62] and (difluoromethyl)phosphonate[84,85] internucleotide bridges have been synthesized by the triester method, and allyl- and (dimethoxytrityl)phosphonates have been obtained by Arbusov reactions.[75]

2. Phosphorothioates/Phosphorodithioates

Phosphorothioates are among the most obvious and thus probably earliest used analogues of naturally occurring phosphates.[86] For example, the antiviral effect of thiophosphate-substituted polyribonucleotides was described back in 1970 by De Clercq and Eckstein.[87] In phosphorothioate oligodeoxynucleotides one of the phosphate oxygen atoms not involved in the bridge is replaced by a sulfur atom, with the negative charge being distributed unsymmetrically and located mainly on sulfur.[88,89] This substitution results in properties such as stability to nucleases, retention of solubility in water, and stability to base-catalyzed hydrolysis, which makes it exceptionally interesting for use in antisense technology. Just like the unmodified oligodeoxynucleotides that have been discussed, the phosphorothioate analogues can be prepared by the three methods of synthesis: the phosphate triester, phosphite triester, and H-phosphonate methods.

The groups of Reese[90] and van Boom[91] have synthesized the phosphorothioates by the triester method using 2,5-dichlorophenyl phoshorodichloridothioate as phosphorylating and coupling component. The activator used by van Boom, 1-hydroxy-6-nitrobenzotriazole (Figure 10), proved to be superior to 1-hydroxybenzotriazole for this. Van Boom reports the yield for a 10-min synthesis cycle as >90% and was able to synthesize hexadecamers in this way. Standard methods were used for cleavage of the support and removal of the protective groups.[91] The corresponding methylphosphonothioates can be obtained analogously by methylthiophosphonic acid benzotriazolide.[92]

The synthesis of diribonucleoside phosphoromonothioates by oxidation of phosphite triesters with sulfur was described first by Burgers and Eckstein,[93] who carried out the oxidation with a 1 M solution of sulfur in pyridine. This method was easily applicable to the synthesis of the 2′,5′-linked oligoribonucleotide phosphorothioate[94] and of dideoxynucleoside phosphoromonothioates.[95] This was extended to oligodeoxynucleotide synthesis on a solid support in 1984.[96,97a] The standard amidite method is used for this synthesis, the only change being oxidation with 0.4 M S_8 in 2,6-lutidine (60 °C, 1 h) or 5% S8 in CS_2/pyridine at room temperature in place of the usual I_2/H_2O[97] (Figure 11). The yield in the subsequent coupling step remains at the same high level.[96] This method is particularly attractive because it can be carried out without extensive alteration of the normal synthesis cycle and is therefore still regarded as the method of choice.[98] Only for the oxidation with sulfur, which is carried out at 60 °C, must the support be removed from the synthesizer. If KSeCN in acetonitrile is used for oxidation in place of sulfur, phosphoroselenide analogues can be obtained.[97a,99] Another advantage derives from the fact that phosphoromonothioate bridges can be introduced in this way at any desired point in oligodeoxynucleotides.[96,100] This is of interest particularly for studies of enzyme mechanisms[86,96] but also for antisense technology.[98] Subsequent oxidation with I_2/H_2O does not seem to cause loss of sulfur. The deprotection of the thiophosphate group from the *O*-methyl ester is carried out at the end of the synthesis with thiophenol. Only in phosphorothioate diesters is the sulfur easily

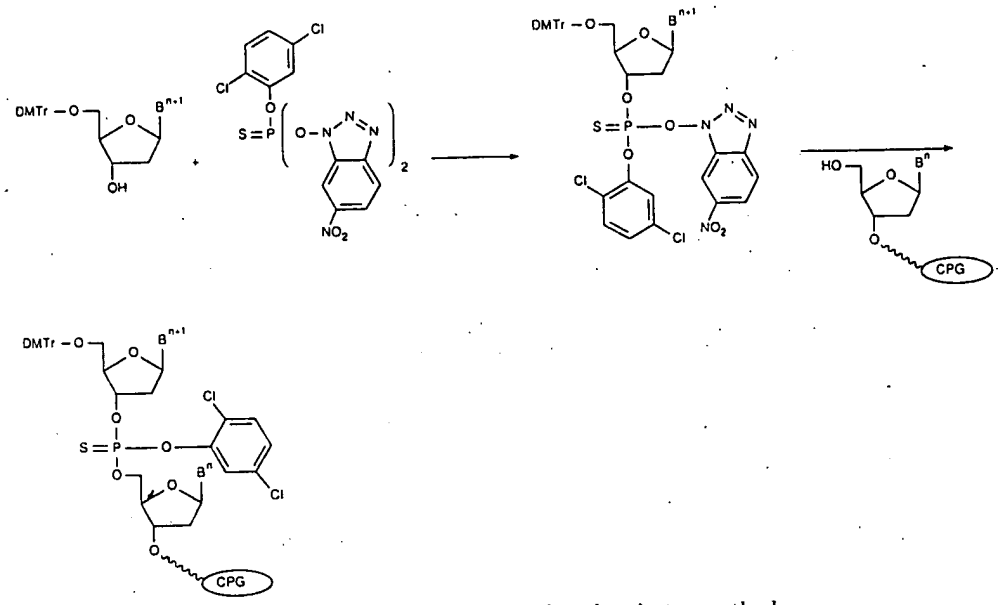


Figure 10. Synthesis of oligonucleotide phosphorothioates by the phosphotriester method.

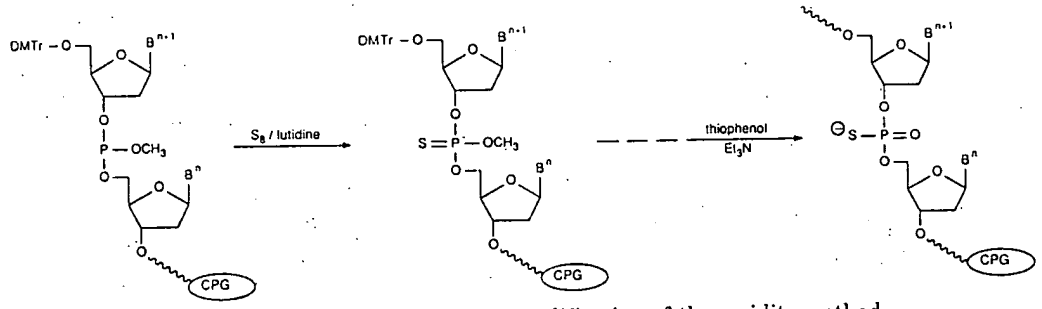


Figure 11. Synthesis of oligonucleotide phoshorothioates by a modification of the amidite method.

replaced by oxygen when oxidation is carried out with 1% I_2 in THF/lutidine/H_2O.[96]

Synthesis of all-phosphorothioate oligodeoxynucleotides is more easily carried out as single-step sulfurization in a modification of the H-phosphonate method.[98] Fujii et al.[101] were the first to describe the oxidation of H-phosphonates with S_8 in pyridine/triethylamine, which was subsequently applied to solid-phase synthesis.[35,98,102] The advantage results from the fact that oxidation with sulfur (0.1 M S in 9:1 CS_2/triethylamine, 2 h) is necessary only once, namely, after synthesis is complete (Figure 12).

The dinucleotide phosphorothioates have, as do the methylphosphonates, a chirality center at the phosphorus. Therefore, they are often used as key substrates for elucidating the stereochemistry of enzymatic reactions.[86,103] The latter can, in turn, be used for configurational assignment: the S_p diastereomers are substrates of nucleases S1 or P1, while the R_p diastereomeric dinucleotides are cleaved by snake venom phosphodiesterase. There is great interest in chromatographic separation of the diastereomeric dimers,[95,96,99,100] spectroscopic characterization,[104] and the possibility of stereospecific synthesis. A first attempt at the synthesis of pure R_p and S_p diastereomers was carried out by Stec et al., who reacted P-chiral phosphoranilidate dimers with NaH/CS_2 by the Wadsworth–Emmons method to give the phosphorothioates.[105,106] Fujii et al.[101] published, in 1986, a method for the stereoselective preparation of the R_p diastereomeric dimers T_ST and dA_ST. This entailed the corresponding aroylphosphonates being reacted with *tert*-butylamine in the presence of DBU and sulfur directly and exclusively to the R_p isomer with a yield of ca. 60% (Figure 13). Cosstick and Williams[107] have supplemented this by describing a synthesis that is based on work by Ohtsuka[108,109] and results in the preferential formation of the S_p diastereomers (Figure 13). This involves the condensation of a β-cyanoethyl S-protected nucleoside 3′-phosphorothioate with a 5′-unprotected nucleoside using 1-(mesitylenesulfonyl)-5-(2-pyridinyl)tetrazole (MSPY). After deprotection an 8:2 excess of the S_p isomer is obtained, but the overall yield of 46% is low.

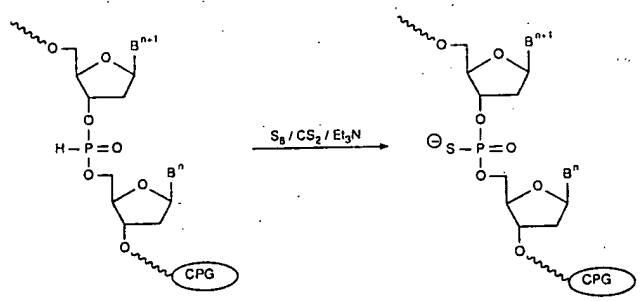


Figure 12. Synthesis of oligonucleotide all-phosphorothioates by a modification of the H-phosphonate method.

The chirality problem can be avoided if the second phosphate oxygen, which is not involved in the bridge, is also replaced by sulfur. Caruthers et al.[110,112] have described the synthesis of such phosphorodithioates. In this, a phosphorothioamidite is condensed with a second

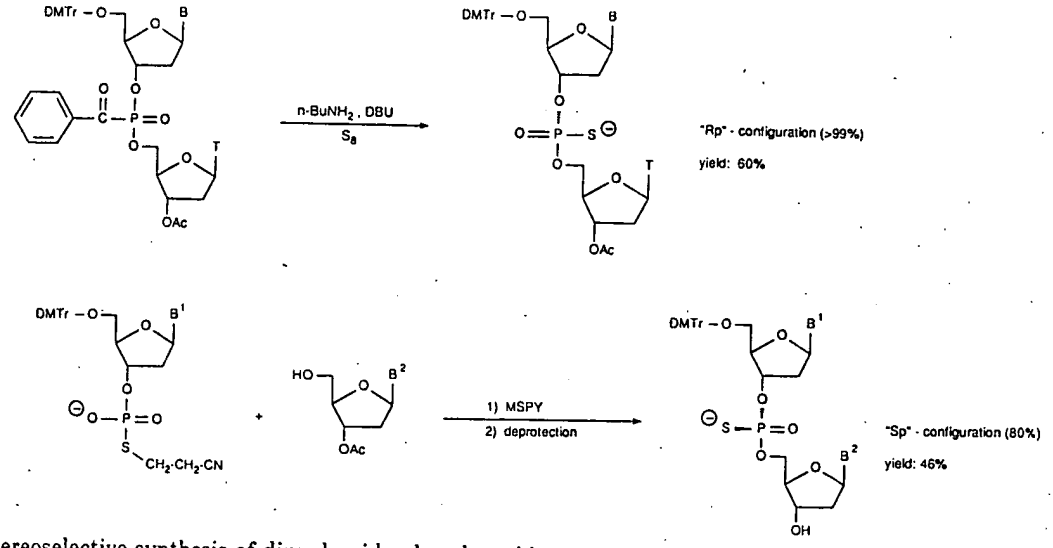


Figure 13. Stereoselective synthesis of dinucleoside phosphorothioates.

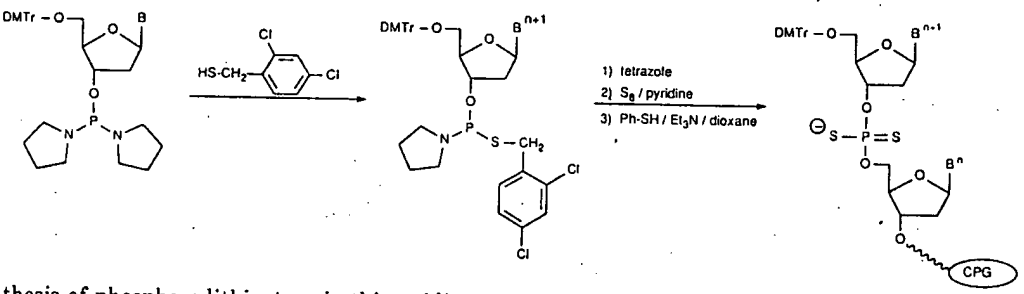


Figure 14. Synthesis of phosphorodithioates via thioamidites.

nucleoside, with tetrazole catalysis, and the resulting intermediate is oxidized with 5% sulfur in pyridine/CS_2 to the phosphorodithioate. This synthesis can also be carried out on a solid support (Figure 14). To ensure high coupling yields (>98%) the phosphorothioamidite must be supplied twice to the solid support in the coupling step. The deprotection of the oligonucleotide phosphorodithioate is carried out in two steps: first the *S*-(2,4-dichlorobenzyl) protective group is eliminated with thiophenol/triethylamine/dioxane, and then the bases are deprotected with concentrated NH_4OH as usual. No hydrolysis to the phosphates or phosphorothioates is observed during this. The synthons used must be in the form of *N,N*-dimethyl- or pyrrolidinylphosphoramidites because the *N,N*-diisopropyl group has proved to be too inert. The advantage of this procedure is that the phosphorodithioate bridge can be incorporated at any desired point in the molecule.

3. *Phosphoramidates*

The phosphoramidates are, like the thiophosphates, an easily obtainable group of nucleotide analogues which can be prepared in various ways from three- and five-valent phosphorus intermediates.

The method of phosphorylating amines by condensation with phosphate diesters in the presence of triphenylphosphine and CCl_4 has often been used to prepare dinucleoside phosphoroamidates[105,106,113] (Figure 15). However, the yields are moderate and vary between 24% (T_NT, *n*-butylamine[113]) and 53% (T_NT, aniline[105]). Yields of 70% (T_NT, *n*-butylamine) are reached only on solid supports.[113]

Other possible syntheses comprise addition of alkyl and aryl azides to phosphite triesters,[113-116] but these

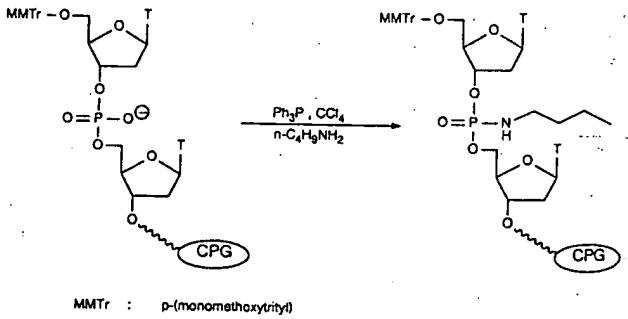


Figure 15. Synthesis of phosphoroamidates by "Appel" condensation.

reactions take place very slowly at room temperature[113,114,116] (several days), and even under more drastic conditions (dioxane, reflux, 4 h) only "moderate" yields (87%[115]) are obtained.

Even less favorable is the preparation of phosphoramidates by nucleophilic substitution of phosphate triesters by alkylamines,[117-119] the yields scarcely exceeding 70% in the most favorable cases.[117]

By contrast, oxidation of dinucleoside H-phosphonates in the presence of amines gives the corresponding dinucleoside phosphoramidates[73,121,122] in high yields.[120] This reaction can easily be extended to polymer-bound di- and oligonucleotide H-phosphonates.[35,102,122,123] The oxidizing agents used for the coupling, which can be carried out with primary and secondary amines, are CCl_4[121,123,124] and I_2[35] (Figure 16).

Besides the described oxidation of H-phosphonates, which results in all-phosphoramidate oligodeoxynucleotides, the oxidation of phosphite triesters with iodine in the presence of alkylamines, which was first described by Nemer and Ogilvie,[73] is particularly at-

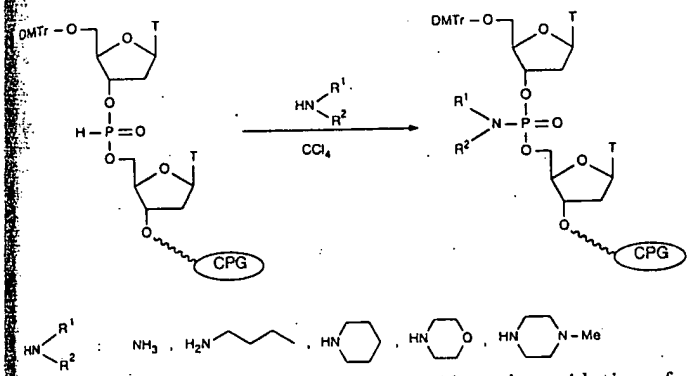


Figure 16. Synthesis of phosphoroamidates by oxidation of H-phosphonates.

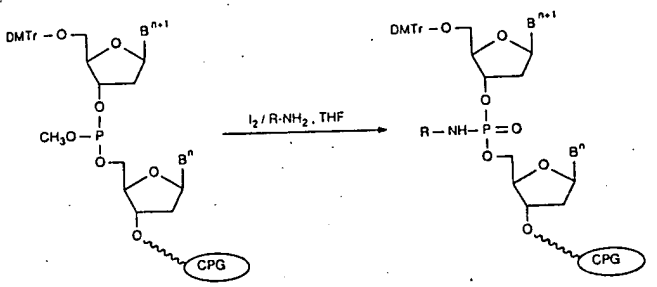


Figure 17. Synthesis of phosphoroamidates by oxidation of dinucleoside phosphite triesters with I_2/alkylamine.

tractive[113] because it can be carried out without expensive alteration in the amidite synthesis cycle.[113] This entails oxidation with 0.1 M I_2 in 2:1 THF/alkylamine, resulting in selective elimination of the methyl protective group used (Figure 17). The yields vary between 50 and 90%.[113] Once again it is possible to incorporate phosphoramidate bridges at any desired point in the oligonucleotide. Deprotection and cleavage of the support are carried out by using *tert*-butylamine in methanol.

None of these methods results in the preferential formation of one diastereomer, but the mixtures in the case of the dimers can easily be separated by chromatography and characterized by spectroscopy.[113] In contrast to the thioates, the diastereomeric phosphoramidates cannot be differentiated by digestion with nuclease P1 or snake venom phosphodiesterase; on the contrary, they are absolutely inert to these enzymes.[113,117] *N*-Alkylphosphoramidates are suitable as functionalizing groups, via the alkyl radical, and can be used to produce oligodeoxynucleotide conjugates, e.g., with intercalating agents.[113,114] These are discussed separately in section II.F.

4. Phosphate Esters

The P(O)-alkyl derivatives, like the alkanephosphonates, differ from the oligodeoxynucleotides in lacking the negative charge on the oxygen in the latter. DNA phosphate triesters have been of interest for a long time as products of alkylating agents,[125] but they acquired additional significance as potential antisense oligodeoxynucleotides.[57,126]

Oligodeoxynucleotide phosphate triesters can be synthesized in a variety of ways. Specific phosphate triesters are produced as intermediates in the phosphate triester method already discussed, as well as in the amidite method. In these cases, the triester serves to

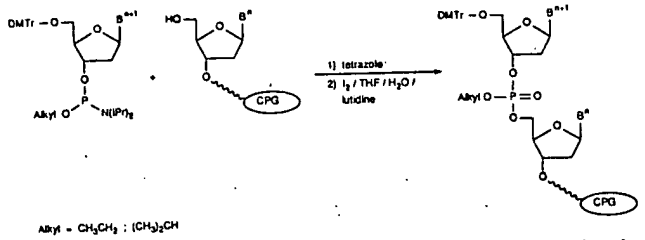


Figure 18. Synthesis of oligonucleotide phosphotriesters by the amidite method.

protect the phosphate and must, accordingly, be easily cleaved. This is why the most suitable groups are those such as *o*-chlorophenyl or 2,5-dichlorophenyl in the triester method and methyl or cyanoethyl esters in the amidite method. However, if the synthesis is to terminate on the triester stage, this restriction does not apply and, accordingly, other esters can be used. The amidite method is widely used to synthesize phosphate triesters from nucleoside dimers and oligomers. Mainly employed for this are the 5′-protected nucleoside 3′-(*O*-isopropyl *N*,*N*-diisopropylphosphoramidites) and their *O*-ethyl analogues[127-132] (Figure 18). However, sterically more demanding esters such as 1,1-dimethyl-2,2,2-trichloroethyl esters[117] have also been prepared. The synthesis is efficient and, moreover, provides the opportunity to replace the I_2/H_2O oxidation by S_8/pyridine and thus obtain phosphorothioate triesters. It is, of course, possible to incorporate both groups at any desired point in normal oligodeoxynucleotides.[129,132] The cleavage of the support and the deprotection of the bases is carried out with 25% NH_4OH at room temperature for 48 h. The ethyl and isopropyl phosphate triesters are stable under these conditions, whereas methyl and cyanoethyl esters are completely removed.[132]

Stec et al.,[133] in 1987, developed a method that combines some advantages of the classical amidite method with some of the H-phosphonate method. This employs 5′-protected nucleoside 3′-*O*-phosphorodimorpholidites as synthons, with hydrolysis to the H-phosphonate per each synthesis cycle (yields between 90 and 99%), and oxidation is then necessary only once, at the end of the synthesis. Alternatively, alcohols can be used for alcoholysis, and the resulting phosphite triesters are oxidized at the end of the synthesis (Figure 19). This method is attractive owing to its versatility.

For the sake of completeness, it may also be mentioned that phosphorothioates can be reacted with 2,4-dinitrofluorobenzene, and the intermediate phosphorofluoridates trapped with alcohols to give triesters.[99]

The problem of the chirality of the phosphorus that has already been mentioned also occurs with the phosphate triesters. Considerable efforts have been made to separate the diastereomers arising due to this chirality center and to characterize them (^{1}H NMR, UV).[99,130,134] The synthesis of ^{18}O-chiral dinucleotides is also based on a separation of this type.[135] It was, of course, interesting in this connection to find a method that allows configurational assignment at the phosphorus irrespective of the nature of the triester. Such a method is based on the stereoselective reaction of a P-chiral phosphothionotriester with *m*-chloroperbenzoic acid to give an *O*-triester on the one hand and with KSeCN as a strongly carbophilic nucleophile to give a

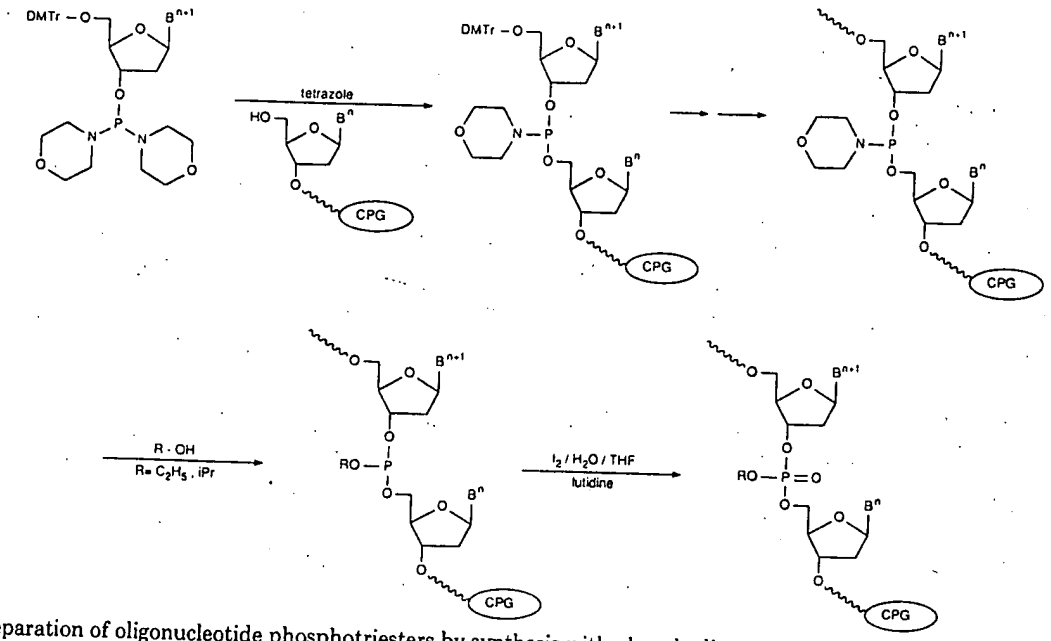


Figure 19. Preparation of oligonucleotide phosphotriesters by synthesis with phosphodimorpholino amidites, alcoholysis, and oxidation.

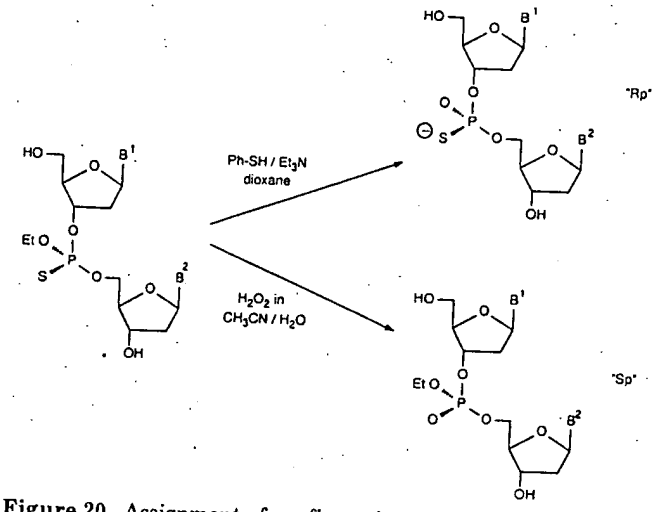


Figure 20. Assignment of configuration of phosphotriesters by stereoselective conversion of thiono triesters to thioates or phosphotriesters.

phosphorothioate on the other hand.[128] An alternative to *m*-chloroperbenzoic acid is H_2O_2 in acetonitrile/water, while an alternative to KSeCN is thiophenol/triethylamine in dioxane[129,132] (Figure 20). Since the phosphorothioates have been thoroughly investigated (see section II.B.2), configurational assignment is not difficult. The dinucleoside monophosphate triesters are resistant to nuclease digestion by snake venom phosphodiesterase and nuclease P1.

As is the case with the oligonucleotide analogues already discussed, stereoselective syntheses are in short supply for phosphate triesters, too. It is worth mentioning the selective formation of S_p diastereomers found by Ohtsuka and Ikehara when the otherwise unselective triester method is carried out with (arylsulfonyl)-5-(2-pyridinyl)tetrazoles as coupling reagents.[108,109,136]

Alkyl esters functionalized in the side chain are suitable as linkers to give oligodeoxynucleotide conjugates, which have been utilized with the alkylating oligodeoxynucleotides as discussed in section II.F.

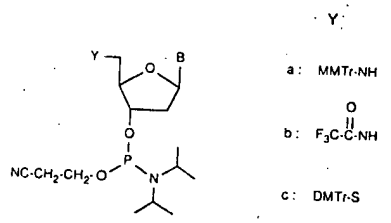


Figure 21. Monomers for incorporating (a) 5′-NH-bridged phosphoramidates, (b) 5′-NH_2 nucleosides at the 5′ end, and (c) 5′-SH-nucleosides at the 5′ end.

5. Replacement of Phosphate Oxygens Involved in the Bridge

Besides the modifications discussed, all of which related to replacement of oxygen atoms not involved in the bridge on the phosphate group, there is also the possibility of replacing one of the two oxygen atoms involved in the bridge. Potential candidates are analogues with NH–, CH_2–, and S–P bridges. This type of linkage results in DNA segments having a number of interesting properties because the chirality problem no longer applies. However, only a few of the conceivable modifications have actually been carried out.

(*a*) *Bridged Phosphoramidates.* The best known of these compounds are the bridged phosphoramidates. These can be prepared most straightforwardly by the amidite method. Bannwarth[137] synthesized the monomers necessary for this (Figure 21) and was able to incorporate them, using the standard method, at any desired point in normal oligodeoxynucleotides, with the coupling yield being >96%. Monomers suitable for incorporation at the 5′ ends of oligodeoxynucleotides have been described by Sproat et al.[138]

Another way of obtaining this class of compounds comprises the azide addition onto phosphite triesters which has already been described. Mag and Engels[139] synthesized and characterized (2D ^{1}H NMR) dinucleoside phosphoramidates in this way, and followed the reaction by IR spectroscopy, specifically of the azide band. There was initial formation of a phosphite imine, followed by a Michaelis–Arbusov-type transformation

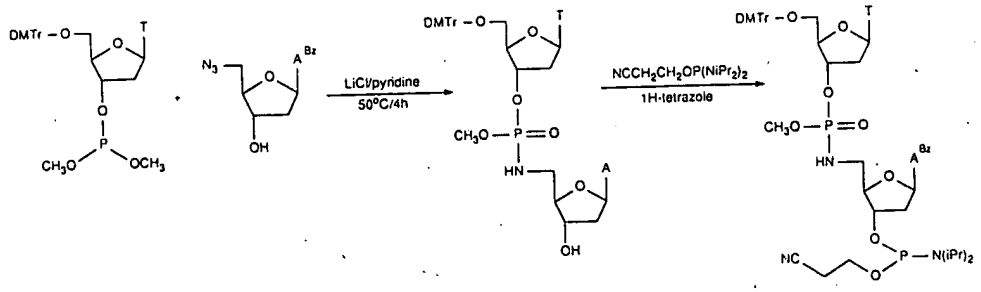


Figure 22. Synthesis of bridged phosphoramidates.

into the phosphoramidate, whose rate is increased by LiCl (Figure 22). These dimers can be converted into amidite units by phosphitylation and reacted further.[139] Trimeric bridged phosphoramidates were synthesized, based on the principle of the azide reaction, for the first time by Letsinger.[116] He was also able to prepare tetramers by a modified triester method.[140]

These compounds can be synthesized not only chemically but also enzymatically. Once again, it was Letsinger[141] who, in a template-controlled polymerase reaction, was able to incorporate 5′-amino-5′-deoxythymidine phosphate via the triphosphate into a DNA fragment. Also worthy of mention is the work by Orgel[142,143] and Shabarova,[144] who were able, likewise in a template-controlled reaction, although without enzyme, to bring about the self-condensation of activated 3′-amino-3′-deoxynucleosides (as 5′-phosphorimidazolides).

The chemical property of the bridged phosphoramidates that must be emphasized is their sensitivity to acids.[145] In 80% acetic acid at room temperature, all the phosphoramidate linkages are cleaved in 6 h, whereas unmodified DNA is not attacked.[137] This can be utilized for cleaving DNA fragments at specific positions under mildly acidic conditions.

(*b*) *Bridged Phosphorothioates.* Although the synthons for 3′-*O*-5′-*S*-thiophosphate oligodeoxynucleotides, namely, the 5′-(*S*-(triphenylmethyl)-thio)-2′,5′-dideoxyribonucleoside 3′-*O*-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidites), can be obtained[146] (Figure 21), these have been incorporated only at the 5′ end of oligodeoxynucleotides.[146] Cosstick and Vyle[147] used a 5′-(monomethoxytrityl)thymidine 3-*S*-thiophosphoramidite to prepare oligonucleotides containing 3′-thiothymidine on a solid support. The intermediate thiophosphites were efficiently oxidized with tetrabutylammonium periodate.

(*c*) *Bridged Methylenephosphonates.* Another modification comprises replacement of one of the oxygen atoms in the bridge by a methylene group. However, this has been achieved to date only in dimers. Thus, Morr et al.[148,149] were able to synthesize the 3′-methylenephosphonate analogues of d(GC) in 80% yield by a method based on triester chemistry (Figure 23).

C. "Dephospho" Internucleotide Analogues

An obvious way of obtaining nonionic oligodeoxynucleotides is to replace the phosphodiester bridge by an entirely different group. Of course, a plethora of replacements is conceivable, but some structural criteria have to be met to ensure that there is still efficient hybridization with the target sequence. This is why the

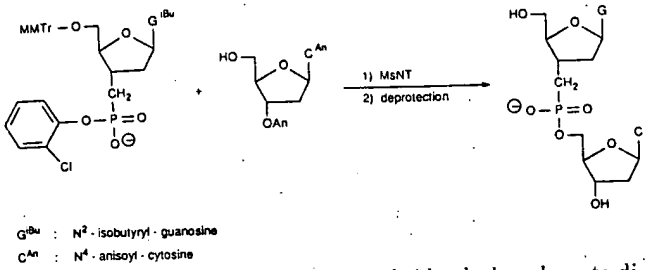


Figure 23. Synthesis of a methylene-bridged phosphonate dinucleoside.

studies described below are primarily aimed at replacement groups that do not change the natural 5′–3′ distance to drastically.

1. Siloxane Bridges

The structures of phosphate and siloxane bridges are very similar because in both cases the central atoms have tetrahedral geometry. Synthesis was initially confined to dimers. This entailed 5′-protected nucleosides being reacted with dichlorodiphenylsilane[150] or dichlorodimethyl- or dichlorodicyclohexylsilane[117] in pyridine and the resulting intermediate being trapped in situ with 3′-protected nucleosides. This resulted in the 3′,5′ dimers in ca. 50% yield. A major problem was the formation of almost 30% of the symmetrical 3′,3′ dimers. On the other hand, the yield of the 3′,5′ compound was considerably increased with imidazole in DMF. Thus, Ogilvie[152] obtained almost 80% 3′,5′ dimers with di-*tert*-butyldichlorosilane and 74% with dichlorodiisopropylsilane and bis((trifluoromethyl)-sulfonyl)diisopropylsilane. He was able to synthesize hexamers using suitable protective groups such as 5′-*O*-dimethoxytrityl and 3′-*O*-levuloyl (the siloxane bridge is sensitive to both bases and acids) (Figure 24).

2. Carbonate Bridges

Oligodeoxynucleotides with carbonate bridges have been described up to the trimers.[153] Whereas phosgene was initially used for the condensation,[154] this was subsequently replaced by activated carbonic esters, with (trichloroethoxy)carbonyl chloride proving most suitable.[153,155] The coupling yields were, however, only ca. 50% because only inadequately protected bases could be employed. It proved to be extremely difficult to remove protective groups in the presence of the carbonic diester.

3. Carboxymethyl Ester Bridges

Polynucleotide analogues (poly-U and poly-T) with carboxyl ester bridges were synthesized as early as 1968 by Jones,[156,157] whose assumption was that these would

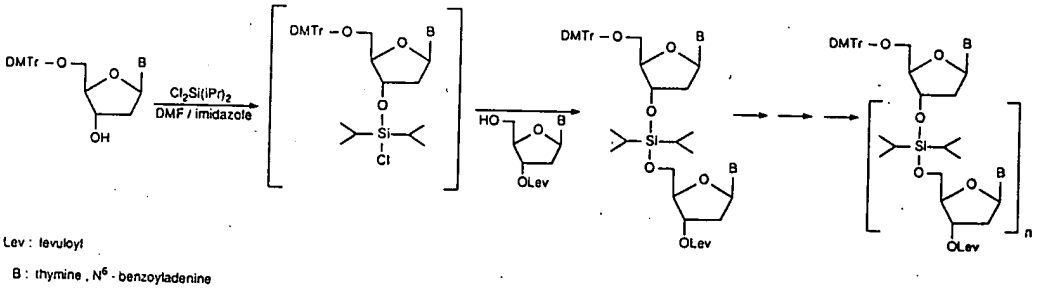


Figure 24. Preparation of siloxane-bridged oligonucleotide analogues.

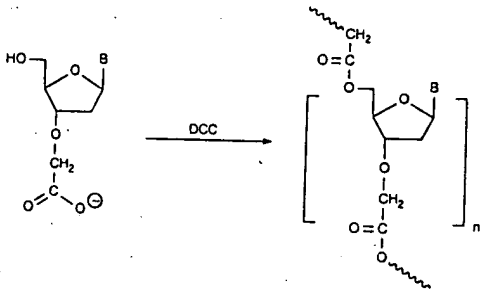


Figure 25. Preparation of poly(3′-*O*-carboxymethyl-2′-deoxynucleoside).

be flexible enough to keep the nucleotide units apart at their natural distance. The synthesis was carried out by polymerization of 3′-*O*-(carboxymethyl)thymidine in pyridine with dicyclohexylcarbodiimide (DCC) and the 5′-O-protected unit as terminator. In 1975 he prepared poly-dA[158] and poly-dC[159] analogues in the same way, with protection being unnecessary for adenine but necessary for cytosine as the 4-*N*-phenoxyacetamide (Figure 25). The resulting oligomers had a mean chain length of 13 bases.[157] In 1971 Jones[159] replaced the polymerization reaction by a stepwise synthesis using suitable protective groups that could be eliminated under nonbasic or only mildly basic conditions because of the sensitivity of the carboxymethyl group to bases (the half-life at pH 7.5 is 7 h[158]). The 5′-OH group was protected with dimethoxytrityl, 2′- and 3′-hydroxyl groups were blocked as isopropylidene and anisylidene compounds, and the carboxyl group was in the form of the 2-cyanoethyl ester. Cytosine required 4-*N*-(dimethylamino)ethylene protection, while the other bases were not acetylated under the reaction conditions. He was able to perform specific syntheses of dimers,[161] trimers,[160] and tetramers.[162]

4. Acetamidate Bridges

Oligodeoxynucleotide analogues with carboxymethyl bridges have two disadvantages: the bridge is unstable under physiological conditions, and the solubility of the compounds is low, which makes it difficult to test their biological activity. It appears possible to solve both problems by replacing the 5′-ester oxygen by an amide, and the acetamidate bridge ought also to provide a suitable internucleotide distance. The dimers were synthesized, in exact analogy to the preparation of the carboxymethyl esters, by condensation of 3′-*O*-(carboxymethyl)-5′-*O*-tritylthymidine with 5′-amino-5′-deoxythymidine and DCC.[163] However, polymerization proved to be far more difficult: condensation of 5′-amino-5′-deoxy-3′-*O*-(carboxymethyl)thymidine with DCC yielded no polymer, only the 3′,5′-bridged lactam as the main product[163] (Figure 26). Polymerization of

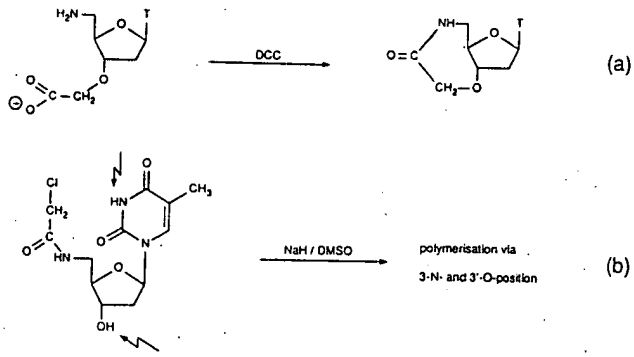


Figure 26. Unsuccessful attempts to prepare acetamidate-bridged polythymidine.

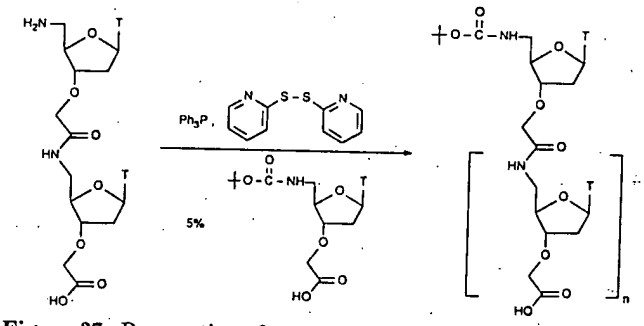


Figure 27. Preparation of acetamidate-bridged oligonucleotides.

5′-(chloroacetamidyl)-5′-deoxythymidine with sodium hydride in DMSO was equally unsuccessful, because alkylation took place not only at the 3′-O but also at the N^3 position of thymidine[164] (Figure 26). On the other hand, when the corresponding 5′-(chloroacetamidyl)-5′-deoxy-4-*N*-acylcytidine was employed, polymerization was successful, but most of the product was lost on deprotection of the base, so that the overall yield was only ca. 1%. Because the lactam formation mentioned above was certainly due to the favorable entropy for intramolecular ring closure, Jones[164] successfully used an appropriate dimer for the polymerization with the aid of triphenylphosphine and 2,2′-dipyridyl disulfide (Figure 27). It was possible to isolate a 54% yield of the highly disperse polymer, whose mean chain length was reported as 10–13. Although the acetamidate bridge is stable over a somewhat larger pH range, unfortunately other desired properties such as stability in water and good hybridization characteristics were not obtained.[163,164] An additional difficulty in the investigations was that the polymer was also adsorbed onto glass and plastic surfaces.

5. Carbamate Bridges

Carbamate bridges were supposed, like the acetamidate bridges, to be superior to the carbonate ana-

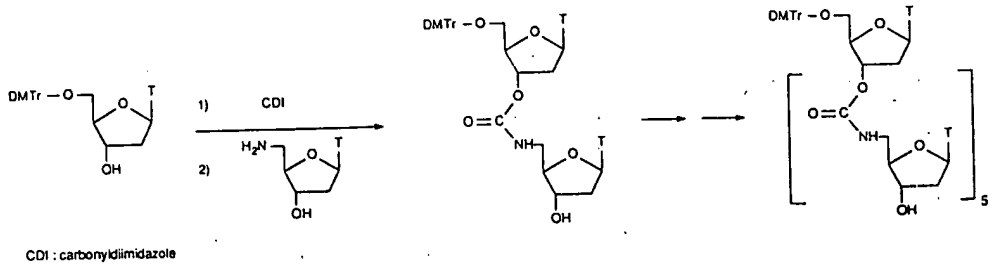


Figure 28. Synthesis of carbamate-bridged oligonucleotides.

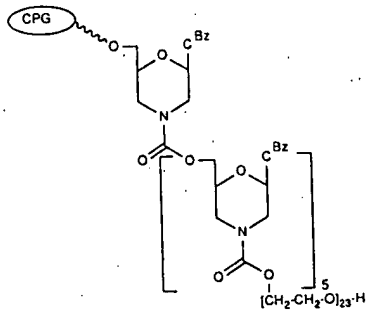


Figure 29. Carbamate-bridged oligonucleotide analogues of the morpholine type.

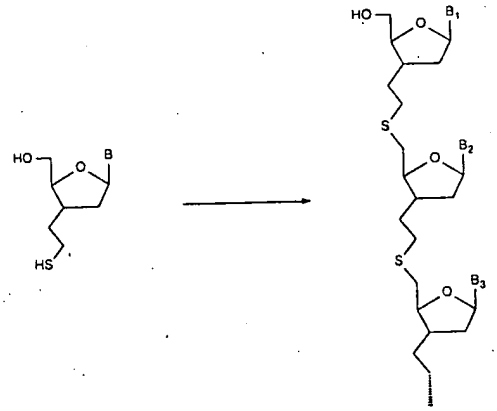


Figure 30. Thioether-bridged oligonucleotide analogues.

logues by having greater pH stability. The first 3′-*O*-5′-*N*-carbamate-bridged dimer of thymidine was synthesized in 1975 by Jones, who obtained it in 38% yield by condensation of the 3′-*O*-(2,2,2-trichloroethyl) carbonate of 5′-*O*-tritylthymidine with 5′-amino-5′-deoxythymidine.[163] Mungall and Kaiser described, in 1977,[165] the synthesis of a trimer in which they used *p*-nitrophenyl esters in place of the trichloroethyl ester. The dinucleoside carbonate that was initially obtained was derivatized with *p*-nitrophenyl chloroformate and again condensed with 5′-amino-5′-deoxythymidine. Not until 1987 (10 years later) was the synthesis of a hexamer described by Coull et al.[166] (Figure 28). They used for this 1,1′-carbonyldiimidazole (CDI) as carbonyl synthon. 5′-*O*-(Dimethoxytrityl)thymidine was alternately reacted with CDI in THF and then, after workup, with 5′-amino-5′-deoxythymidine in pyridine. The hexamer was obtained in 40% yield. Like the compounds mentioned above, its solubility in water as well as in organic solvents is low, and it adsorbs onto glass surfaces. It, like the acetamidate analogues, does not enter into base pairing with the natural compounds.[166] The work of Stirchak et al.[167a] contrasts with these results. They synthesized a corresponding cytosine oligomer from dimer blocks which they condensed with bis(4-nitrophenyl) carbonate in DMF/triethylamine. The final study to be mentioned here is one by Summerton[167b,c] which shows the way to an entirely new class of compounds. Ribonucleosides were used as the starting material for the synthesis of *N*-trityl-6′-(*p*-nitrophenyl) carbonate protected monomer units of the morpholine type. Coupling in DMF without catalyst was applied several times and resulted in the formation of polycarbamate compounds. The solubility of the product in water was improved by terminal conjugation via a carbamate with polyethylene glycol (Figure 29).

6. *Thioether Bridges*

The synthesis of thioether-bridged oligodeoxynucleotides as neutral, nonhydrolyzable analogues from 3′-deoxy-3′-(2-mercaptoethyl)nucleoside synthons was reported by Kawai and Just[168] (Figure 30), but no experimental details were given.

7. *"Plastic" DNA*

Besides replacement of the phosphate group, there is also the possibility of replacing sugar and phosphate residues by a synthetic polymer and thus obtaining "plastic" DNA with new and interesting properties. A large number of such analogues have already been prepared. These include poly(*N*-vinyl), poly(meth-

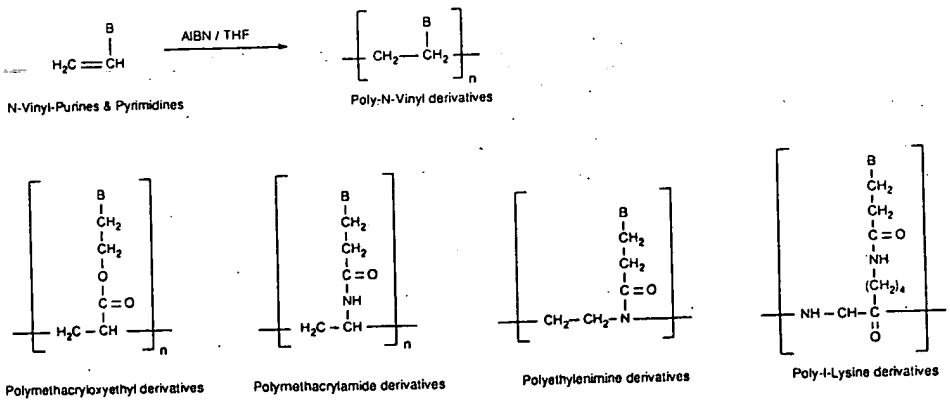


Figure 31. Examples of "plastic" DNA.

acryloxyethyl), poly(methacrylamide), poly(ethylenimine), and poly(amino acid) derivatives, to mention just a few (Figure 31). The synthesis and use of these compounds have been reviewed in several excellent articles, some of recent date.[169-171] Hence, only the poly(*N*-vinyl) derivatives are to be dealt with by way of example at this point, these having been synthesized and very carefully tested for their biological and antiviral activity by Pitha.[171] They were prepared from the *N*-vinyl monomers[172] by radical polymerization in DMSO,[173] DMF,[174] or THF.[175] It was possible in this way to obtain poly(1-vinyluracil), -(9-vinyladenine), -(1-vinylcytosine), and -(9-vinylhypoxanthine). The solubility in water of all of them was poor in the neutral range but good at basic pH. The mean chain length was about 300,[175] so it is appropriate to refer to these molecules as polymers.

D. Oligodeoxynucleotides with Modified Nucleoside Units

1. α-Anomeric Nucleoside Units

In virtually all naturally occurring nucleosides and nucleotides the glycosidic linkage is in its β-anomeric form. On the basis of Dreiding stereomodels Sequin[176a] postulated that the secondary structure of oligo-α-deoxynucleotides based on the β-DNA structure ought to resemble that of the natural β-anomeric DNA. He also predicted that such α-anomeric oliodeoxynucleotides ought to form a helix with a complementary β strand, and the two strands ought to have parallel polarity. Both postulates were later confirmed by Imbach.[176b] The first synthesis of α-anomeric dimers was in 1973 by Holy[177] (βU-αU, βG-αU). α-Anomeric nucleosides were prepared first by Robins[178] in 1969 and subsequently by others.[179] In 1974 Sequin[180] published the synthesis of the four isomeric dithymidine monophosphates βT-βT, αT-βT, βT-αT, and αT-αT, which he obtained by phosphotriester chemistry. The α-anomers proved to be astonishingly stable to nucleases, and Imbach's group took up this modification to use it in antisense oligonucleotides.[181] They synthesized hexameric α-anomeric oligodeoxynucleotides, initially consisting only of αdC and αT,[182] but then from all four α-anomeric deoxynucleotides,[183] using the classical triester method. The customary protective groups were employed, except that guanosine was used as 2-*N*-palmitoyl-6-*O*-(diphenylcarbamoyl)-α-2′-deoxyguanosine. No particular differences from the chemistry of the β-anomers were noticed during the synthesis. The coupling yields were between 80 and 99%. The hexamer consisting of αdC and αT was characterized by ^{1}H NMR spectroscopy,[182] from which it emerged that both thymine and cytosine assume an anti conformation and that the preferred conformation at the sugar is $C_{3'}$-endo (Figure 32). However, the ^{1}H NMR data did not allow the polarity of a hybrid with the complementary oligo-β-deoxynucleotide to be determined. Later on[184] it was possible to show by ^{1}H NMR that an α,β hybrid had parallel polarity. Subsequent investigations on oligo-α-deoxynucleotides with all four bases[185] confirmed that they are present in the anti conformation, but the ring conformation of the sugar was $C_{3'}$-exo. The oligo-α-deoxynucleotides displayed the expected stability toward phosphodiesterases

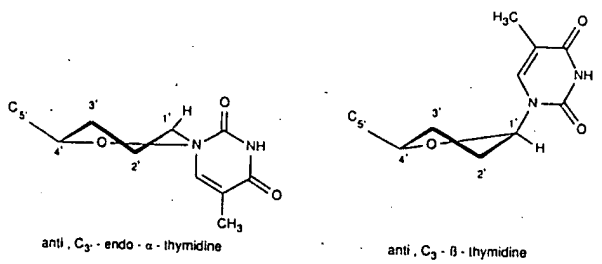


Figure 32. Preferential conformations of α- and β-thymidine.

both from snake venom and from calf spleen. Finally, in 1988 Imbach et al.[186,187] reported the successful solid-phase synthesis of α-anomeric oligodeoxynucleotides by the amidite method. This synthesis was completely analogous to that of the β-anomers except for the use of the 2-*N*-palmitoyl in place of the 2-*N*-isobutyryl protective group on deoxyguanosine. Thereby, α-deoxynucleoside 3′-*O*-((methyl) diisopropylphosphoramidites) were used. The mean coupling yield for a 12-min synthesis cycle was 99% for a pentadecamer and 98.3% for an eicosamer. A similar approach has been reported by Thuong et al.,[188] but they used cyanoethyl-protected phosphoramidites. Imbach[189] and Thuong and Hélène[188,190-193] have reported the synthesis of α-anomeric oligodeoxynucleotides and their linkage with intercalating agents and photo-cross-linkers. This is dealt with in more detail in section II.F.

2. Base-Modified Oligodeoxynucleotides

Apart from the natural bases adenine, guanine, cytosine, and thymine, also incorporated in oligodeoxynucleotides have been other, less common or even purely synthetic bases. These efforts were usually aimed at testing the effect of other modifications, such as methylation of bases, on the action of DNA-modifying enzymes, examining alterations in hybridization characteristics, or even introducing, via reactive groups, covalent bonds between hybridizing DNA strands. In particular, interest has been directed at nucleosides that hybridize nonspecifically, such as inosine, which is located in the "wobble" position of the anticodon in some transfer RNAs. DNA probes with base modifications of this type can be used as an alternative to oligonucleotide mixtures for locating sequences that are ambiguous owing to the degeneracy of the genetic code. Deoxyinosine (Figure 33) has been incorporated in oligodeoxynucleotides for this reason. This was carried out both by the amidite method[194] and by the phosphotriester methods.[195,196] Hypoxanthine, the base of inosine, needs no protection during this. Seela succeeded in synthesizing some isosteric purine 2′-deoxyfuranoside analogues such as 2′-deoxytubercidin and 8-aza-7-deaza-2′-deoxyadenosine (Figure 33) and in incorporating them into oligonucleotides via the 3′-phosphoramidites.[197,198] The amidite method has also been used to introduce 2′-deoxynebularine and 2′-deoxyxanthosine (Figure 33) into oligonucleotides,[199] it being necessary to protect deoxyxanthosine with the 6-*O*-(2-(*p*-nitrophenyl)ethyl) group which was introduced by Pfleiderer[200] into nucleoside chemistry. These nucleosides were also intended to act as nonspecific hybridization partners.

Enzymatic incorporation of base-modified nucleosides into oligodeoxynucleotides is also possible, such as the incorporation of 2-amino-2′-deoxyadenosine (Figure 33)

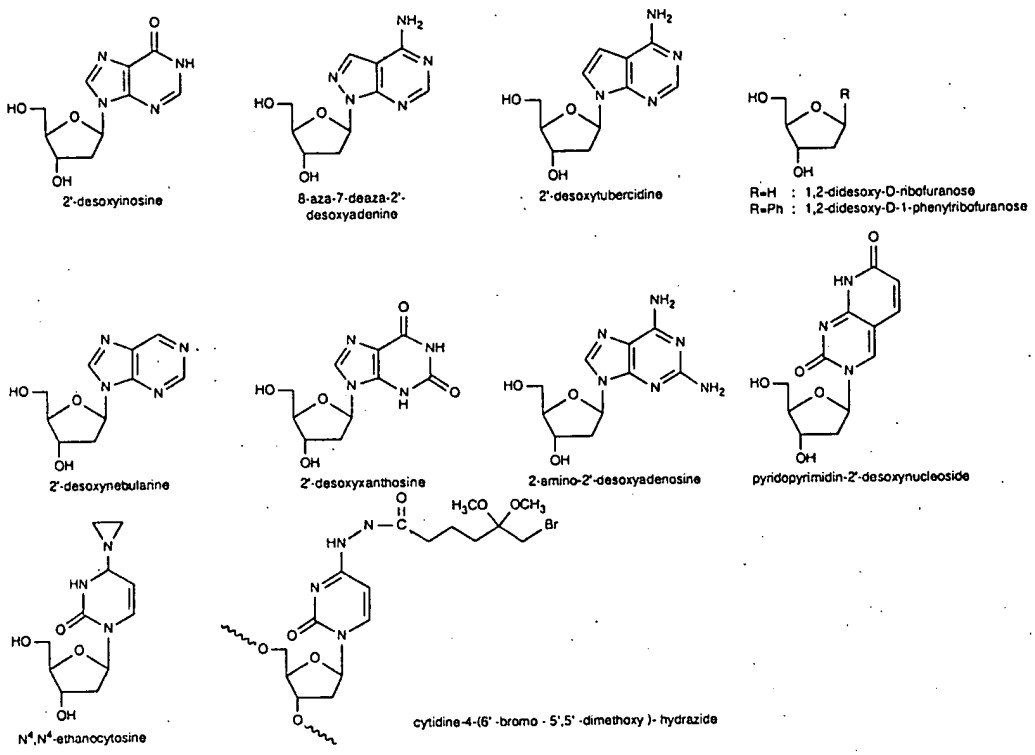


Figure 33. Base-modified nucleosides.

via the triphosphate in a reaction catalyzed by Klenow polymerase.[201,202] However, the modifications have not been confined to isosteric compounds, and in some cases drastic alterations have been made. Millican et al.[203] used 1,2-dideoxy-D-ribofuranose and 1,2-dideoxy-1-phenylribofuranose units (Figure 33), which they introduced into oligonucleotides via phosphotriester synthesis. As expected, the duplexes are less stable with such compounds than those between completely complementary strands. Interesting properties are also shown by the fluorescent 3-(β-D-2′-deoxyribofuranosyl)-2,7-dioxypyrido[2,3-*d*]pyrimidine (pyridopyrimidinedeoxynucleoside) incorporated likewise by phosphotriester chemistry into oligodeoxynucleotides by Ohtsuka[204] (Figure 33). Whereas all the previously mentioned nucleoside analogues could be used in the synthesis without altering the customary reaction conditions, particular care was necessary with the deprotection of oligonucleotides containing pyridopyrimidine (NH_4OH, room temperature, 24 h).

Also of interest for use in antisense oligonucleotides are units that have groups able to react with the target sequence. Ideally, this reaction ought to take place only after the hybridization has occurred. Matteucci and Webb achieved such "hybridization-triggered cross-linking" by incorporating N^4,N^4-ethano-5-methylcytosine (C^e) (Figure 33) via its 3′-phosphoramidite.[205] However, because C^e is not stable to the normal deprotection conditions (NH_4OH, 60 °C, 5 h), 9-fluorenylmethoxycarbonyl was used to protect the exocyclic amino groups, since it can be eliminated under mild conditions (0.5 M DBU, 18 h, room temperature). It was possible in this way to incorporate C^e at any desired point in 20-mers. A modification of this method is reported by Benkovic et al.[206] They prepared the 4-triazole derivative of 5′-(dimethoxytrityl)thymidine, which they incorporated into oligonucleotides and then reacted it with aziridine to N^4,N^4-ethanol-5-methylcytosine (C^e). A similar approach was taken by Bartlett and Summerton,[207] who introduced 6-bromo-5,5-dimethoxyhexanohydrazide in the C^4 position of cytidine, which then became able to alkylate guanosine and thus to cross-link.

Finally, mention may be made of the synthesis of *O*-methyl- and *O*-alkyl-modified bases and their incorporation in oligodeoxynucleotides.[208,209] This was carried out without special precautionary measures in the usual synthesis cycles. These compounds are mainly used for mechanistic studies.

E. Oligoribonucleotides and 2′-Modified Derivatives

1. Oligoribonucleotides

The methods for synthesizing oligoribonucleotides, which originated in the 1960s,[210] have been reviewed by Ohtsuka and by Reese,[211] and thus only a few recent developments will be discussed here. The main problem in the synthesis is in finding a suitable protective group for the 2′-hydroxyl group, which must be compatible with that in the 5′ position. The 2′ protective group must be stable under the conditions of synthesis, especially when the 5′ protective group is removed, and must be amenable to elimination at the end in such a way that there is no possibility of isomerization or cleavage of the phosphate diester bridge.

The usual procedure[212,213] of using two protective groups with different acid stabilities has recently been refined by Reese,[214] who used the 1-(2-chloro-4-methylphenyl)4-methoxy-4-piperidinyl group (Ctmp) to protect the 2′ position (Figure 34). The 9-phenyl-9-xanthenyl group (pixyl) was introduced in the 5′ position, being somewhat more labile than the 4,4′-dimethoxytrityl group. The 2′-acetal protective group is completely stable under the conditions necessary to eliminate the pixyl group, but can itself be easily re-

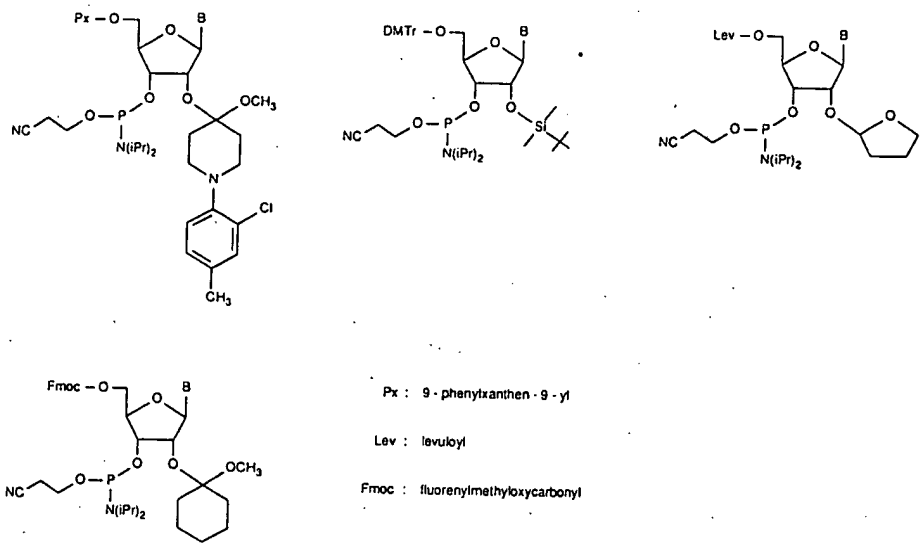


Figure 34. Synthons for the solid-phase synthesis of oligoribonucleotides with various protective groups.

moved at pH 2 in water. Reese was able, using amidite chemistry, which has also become widely used in RNA synthesis, to prepare 19-mers with mean coupling yields of 95%.[213]

Another approach is the use of *o*-nitrobenzyl as the 2′ protective group, which can be removed by photolysis at pH 3.5. In combination with acid-labile 5′ protective groups it has been employed for oligoribonucleotide synthesis by the amidite methods,[215] the H-phosphonate methods,[216] and the phosphate triester methods.[217] Ogilvie[218] used the *tert*-butyldimethylsilyl group, which can be cleaved with fluoride ions, to protect the 2′ position with great success. Thus, he was able to synthesize a 77-mer by the amidite method. Finally, mention may be made of two interesting developments: protection of the 2′ position with acid-labile groups such as the tetrahydrofuranyl and 4-methoxytetrahydropyranyl groups and the use of the levuloyl group in the 5′ position, which can easily be removed by hydrazinolysis[219] (mean coupling yield/synthesis cycle ca. 90%), and the Fmoc protective group,[220] which can be removed under mild basic conditions with 0.1 M DBU in acetonitrile (mean yield/synthesis cycle ca. 96%) (Figure 34). Under these conditions there is probably some elimination of cyanoethyl groups, which results in side reactions.

Another important aspect of the synthesis of oligoribonucleotides is the use of enzymes. A rapid and efficient method for small amounts of unmodified oligoribonucleotides is in vitro transcription using SP6 or T7 RNA polymerase and synthetic DNA.[221] Small segments can be linked together using T4 RNA ligase,[222] the smallest substrates being nucleoside 3′,5′-diphosphates. This method enabled relatively large RNA fragments to be synthesized quite early on.[211]

2. 2′-Modified Oligoribonucleotides

The instability of oligoribonucleotides under physiological conditions led to the synthesis of their 2′-modified analogues. These modifications were confined to the 2′-*O*-methyl derivatives, which also occur naturally and whose monomers were synthesized in 1965 by Furukawa et al.[223] This class of compounds is attractive for antisense applications because ribonucleosides are much less costly as starting materials than their deoxy analogues and because the thermal stability of the hybrids of 2′-*O*-methylribonucleotides with complementary RNA is much greater than that of corresponding DNA–RNA duplexes.[224] The synthesis of 2′-*O*-methylribonucleotides and oligodeoxynucleotides differ only slightly. The former can be prepared just as well by the phosphotriester methods,[224] the amidite methods,[225] and the H-phosphonate methods,[226] the only limiting factor being the availability of monomeric units. Nor did the coupling yield differ in any case from that of the deoxy analogues, although the use of bulky protective groups, such as the *tert*-butyldimethylsilyl group, in the 2′-*O* position results in a drastic reduction in the yields.[228] Sproat et al.[227] employed 5-(4-nitrophenyl)-1*H*-tetrazole (0.1 M in acetonitrile) as activator in the amidite method and achieved yields of >99% with coupling times of 6 min, whereas this time was far too short with 0.5 M 1*H*-tetrazole alone. We have had good results with the protocol of Pon.[229]

The synthesis of 2′-*O*-methylribonucleotide phosphorothioates to inhibit HIV, via corresponding H-phosphonates, has been described and is entirely analogous to the deoxynucleoside phosphorothioates.[226]

F. Oligodeoxynucleotide Conjugates

Oligodeoxynucleotides can be covalently linked to a wide variety of molecules. In the context of antisense technology, this has usually been done to improve transport and hybridization properties of the oligonucleotides. Conversely, conjugates can also be regarded in such a way as to introduce a specificity into otherwise unspecific DNA-binding molecules by covalently linking them to a selectively hybridizing oligonucleotide. In the following, conjugates are differentiated according to the position of their linkage to oligonucleotides and according to their function. This covalent linkage is usually brought about at a 5′ end with phosphorylating reagents and at a 3′ end via modified linkers.

1. Conjugation via the 5′ End

Covalent linkage from the 5′ end of oligodeoxynucleotides is particularly attractive because it can be carried out in the solid-phase synthesis in a similar

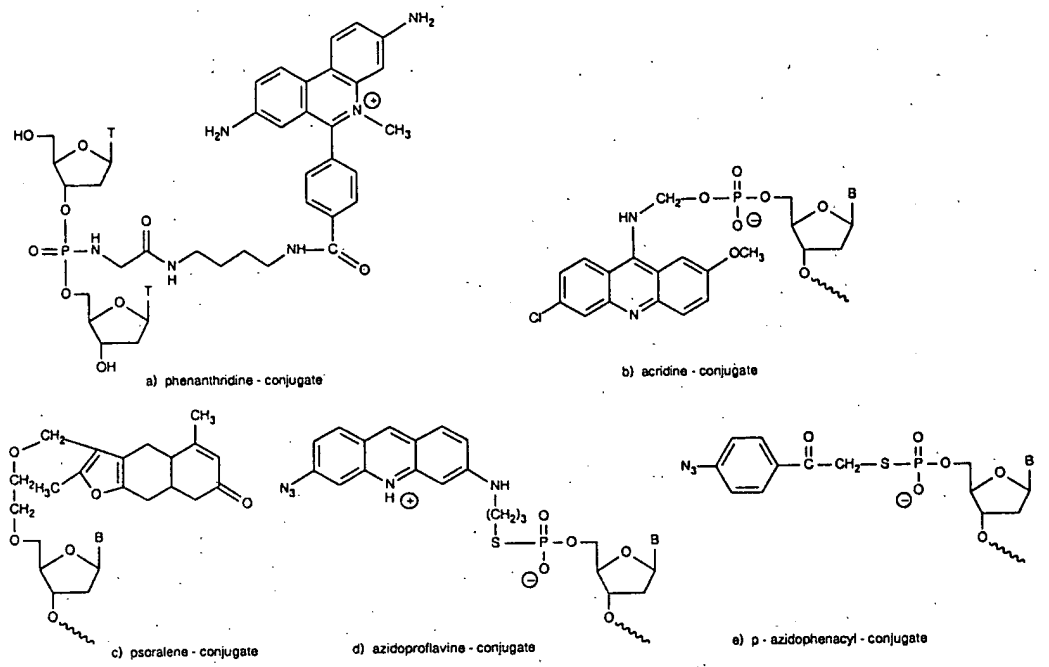


Figure 35. Conjugates of oligonucleotides with intercalating agents and cross-linkers.

manner to the 5′-phosphorylation of oligonucleotides that we have described[32] provided that the group which is to be linked can be derivatized as a phosphoramidite, H-phosphonate, or phosphate and withstands the coupling and deprotection steps.

(*a*) *Intercalating Agents.* The idea of linking an intercalating compound to a "recognition sequence", namely, an oligonucleotide, was described first by Letsinger[230] but was achieved only for a dithymidylate linked via a phosphoramidate bridge to phenanthridine (Figure 35). There have been a number of papers in which phosphotriester or amidite chemistry has been used for covalent attachment of intercalating agents, usually acridine derivatives, to the 5′ end of modified and unmodified oligodeoxynucleotides.[231-234] Thus, 2-methoxy-6-chloro-9-((5-hydroxypentyl)amino)acridine has been linked via its phosphoramidite to oligodeoxynucleotides, oligodeoxynucleotide methylphosphonates, and α-anomeric oligodeoxynucleotides[188,235,236] (Figure 35). The same intercalating agent had previously been incorporated by using phosphotriester chemistry.[237,238] The work of Jäger et al. should be mentioned once again in this connection; they replaced the I_2/H_2O oxidation in the amidite method by an I_2/1,5-diaminopentane oxidation and thus were able to introduce an alkylamino linker via the resulting phosphoramidate and, via the former by alkylation, an acridine derivative at any desired point in the molecule.[113]

(*b*) *Cross-Linkers.* The introduction of cross-linkers into oligodeoxynucleotides via modified bases has been discussed in a previous section (II.D). Cross-linkers can also be linked to the 5′ position by a variety of methods.[232-234] Thus, psoralen derivatives (Figure 35) have been incorporated in oligonucleotides and oligonucleotide methylphosphonates via amidite chemistry[239] and phosphotriester chemistry.[240] Hélène et al. introduced azidophenacyl[241,243] (Figure 35), proflavine,[242] and azidoproflavine[190] (Figure 35) to the 5′ position by first converting the oligonucleotide into the 5′-thiophosphate, which could then be alkylated via a bridge

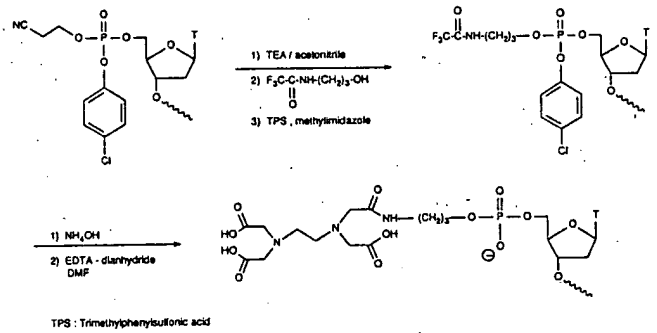


Figure 36. EDTA derivatization of oligonucleotides.

with the particular cross-linker (3-azido-6-((3-bromopropyl)amino)acridine[190] or *p*-azidophenacyl bromide[241,243]).

(*c*) *Artificial Endonucleases.* The term "artificial endonucleases" is intended to comprise those conjugates whose nuclease component is able as such to cleave DNA nonspecifically and acquires a specificity by covalent linkage to the oligonucleotides. This class of compounds includes, in particular, metal complexes such as EDTA–Fe(II), *o*-phenanthroline–Cu(I), or porphyrin–Fe(II), but nonspecific nucleases have also been linked to oligonucleotides for this purpose.

Boutorin et al.[244] derivatized an oligothymidylate with EDTA by first introducing an alkylamino group by condensation with aminopropanol and then, after deprotection, acylating it with EDTA dianhydride (Figure 36). Nuclease activity (local production of hydroxyl radicals) is achieved by incubation with Fe(II). Orgel et al. followed a similar procedure but they used ethylenediamine as linker in place of aminopropanol.[245,246] Ethylenediamino bridges have also been employed by Miller and Ts'O for the EDTA derivatization of oligodeoxynucleotide methylphosphonates.[247]

The work of Dervan should also be quoted at this point: he achieved EDTA linkage by modification of thymine in the 5 position.[248,249] This entailed thymidine-5-propionic acid 2-aminoethylamide being synthesized first and then acylated with EDTA. The ad-

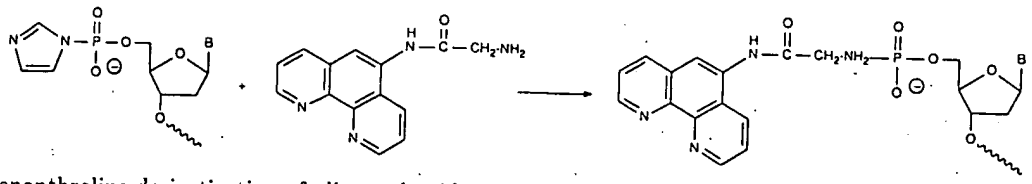


Figure 37. Phenanthroline derivatization of oligonucleotides.

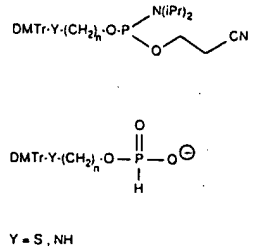


Figure 38. Synthons for the amino- and thiofunctionalization of oligonucleotides.

vantage of this synthon is that it can be incorporated at any desired point in the oligonucleotide.

The conjugation with phenanthroline was carried out in a similar manner to the EDTA derivatization. Chen and Sigman[250] converted their 5′-phosphorylated oligonucleotide initially into the imidazolide, which they condensed with 5-(glycylamido)-1,10-phenanthroline (Figure 37). Hélène et al.[251] chose derivatization via a 5-hexamethylene–thiophosphate linker which was alkylated with 5-(iodoacetamido)-1,10-phenanthroline.

Derivatization with porphyrins and enzymes in the 3′ position is described in section II.F.2.

(*d*) *Lipophilic Carrier and Peptide Conjugates.* The transport of oligodeoxynucleotides into cells has been facilitated by linking them to lipophilic carriers and peptides. Goodchild et al.[252a] described the incorporation of long-chain alcohols as phosphate esters in the 5′ position of oligonucleotides. This can take place at the end of the solid-phase synthesis by use of an appropriate phoshoramidite. Several groups have now synthesized cholesterol conjugates of oligonucleotides;[253] however only one example has been reported in the literature up to now by Letsinger et al.[252b]

Universal methods for functionalizing oligonucleotides with amino or mercapto groups have been described by Sinha and by Connolly,[254] who used H-phosphonate and amidite chemistry to attach aminoalkyl and mercaptoalkyl linkers to the 5′ end (Figure 38). A large number of derivatives, such as dyes or other nonradioactive markers, could be introduced via these linkers. Simple linkage with polylysine via a maleimide has also been described. Polylysine is intended as a carrier for oligodeoxynucleotides.[255]

The synthesis of nucleopeptides via the phosphate esters of the side chains of tyrosine and serine has been described by van Boom.[256] Because of the lability of the serinyl–nucleotidyl phosphodiester bond to bases, special protective groups are required for the exocyclic amino groups. Van Boom used the di-*n*-butylformamidino and the 2-nitrosulfenyl groups, which can be eliminated under milder conditions. Diribonucleosidephosphoro(P–N)amino acid derivatives were first described by Juodka and Smrt[119] via the synthesis of phosphoramidates.

2. Conjugation via the 3′ End

(*a*) *Intercalating Agents.* The synthesis of conjugates of intercalating agents with oligonucleotides via the 3′ position has been extensively investigated by Hélène et al. The intercalating agent mostly used was 2-methoxy-6-chloroacridine, which has already been described. Whereas 5′ derivatization is primarily carried out in solid-phase synthesis using amidite chemistry, that in the 3′ position mainly employes triester chemistry (Figure 39).[257–262] 3′ conjugation of modified oligonucleotides such as methylphosphonates and methyl esters[263] has also been described. Gautier et al.[189] have reported the linkage of α-anomeric oligonucleotides with oxazolopyridocarbazole via the 3′ end using an aminoalkyl linker.

(*b*) *Alkylating Oligonucleotides.* The idea of specific and irreversible modification of DNA by cross-linking was put into practice as much as 20 years ago by Grineva et al.[264] They used for this a diribonucleotide that was functionalized at the 3′ end via an acetal linkage with an aromatic (2-chloroethyl)amino group (Figure 40). The neighboring group participation of the nitrogen means that the (chloroethyl)amino groups are particularly susceptible to alkylation and thus cross-linking (cf. mustard gas). This mode of substitution was subsequently extended to oligonucleotides with a 3′-ribonucleoside residue and has been widely used in antisense technology.[265–269] The alkylation reaction appears to be "hybridization triggered" (cf. section II.E.2), because no self-alkylation takes place. Alkylating groups have also been attached in the 5′ position via phosphoramidate linkages (Figure 40).[266]

(*c*) *Artificial Endonucleases and Enzyme Conjugates.* Of course, it is also possible to attach DNA-cleaving groups at the 3′ end of oligonucleotides, although but few examples of this are known. Hélène et

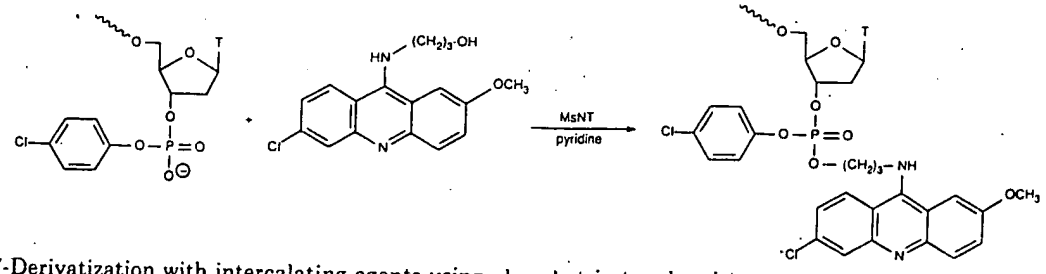


Figure 39. 3′-Derivatization with intercalating agents using phosphotriester chemistry.

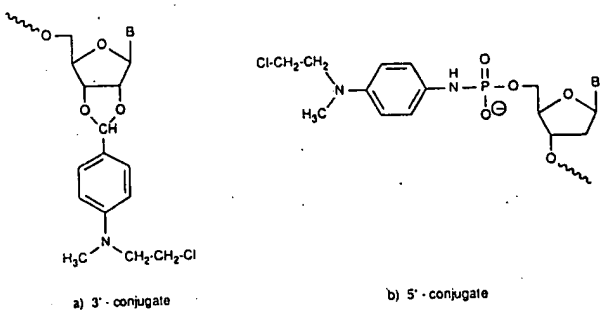


Figure 40. Alkylating oligonucleotides.

al.[270,271] carried out porphyrin derivatization of oligonucleotides in the 3′ position. This entailed a methylpyrroporphyrin ethyl ester being initially reacted with amino-1-hexanol as linker and then coupled using triester chemistry to the 3′ position of an oligothymidylate (dT_7). After metalation with Fe(II) the conjugate displayed nuclease activity.

Oligonucleotide–enzyme conjugates have also been synthesized with a view to converting nonspecific nucleases into specific endonucleases by attaching a selectively binding oligonucleotide. Two examples of this type of modification are known from the literature. Schultz et al.[272,273] succeeded in covalently bonding staphylococcal nuclease to a 15-mer oligonucleotide. This was performed very elegantly via a disulfide exchange. First a 3′-*S*-thiopyridyl oligonucleotide was synthesized, and a lysine was replaced by a cysteine in the nuclease by site-specific mutagenesis, which produced a unique free mercapto group. The two molecules then reacted to give the desired conjugate (Figure 41). Jablonski et al.[274] synthesized an oligonucleotide conjugate with alkaline phosphatase. Linkage took place via a 2′-deoxyuracil-5-aminoheptyl unit with glutaraldehyde. The enzyme activity was retained in both cases.

(*d*) *Oligonucleotide–Peptide Conjugates.* To improve the transport properties of oligonucleotides Lebleu et al. synthesized oligonucleotide–polylysine conjugates.[255,275,276] Oligonucleotides were first provided with a 3′-terminal ribose unit using T4 RNA ligase. This ribose was oxidized with periodate, condensed with a 6-amino group of polylysine, and reduced with $NaBH_3CN$ to the *N*-morpholine conjugate (Figure 42). It is simpler to synthesize chemically a "chimeric" oligodeoxyribonucleotide that contains at the 3′ end a ribonucleoside, the solid support (controlled pore glass, CPG) being loaded, for example, with 5′-*O*-(dimethoxytrityl)-N^4-benzoylcytosine 2′(3′)-succinate, the free 2′(3′)-OH group being acetylated and then the standard synthesis being carried out.[277]

On the other hand, the successive solid-phase synthesis of oligodeoxynucleotides with a 3′-peptide unit, as described by Haralambidis et al.,[278] is somewhat more general. After the peptide chain has been synthesized on the support by the Fmoc method, a 4-hydroxybutyryl linker is attached, and then the oligodeoxynucleotide synthesis is carried out. The conjugate is deprotected at the end of the solid-phase synthesis.

(*e*) *Terminal Transferase.* Terminal deoxynucleotidyl transferase catalyzes the addition of deoxynucleotides at the 3′ end of DNA molecules.[279] Both single- and double-stranded DNA with at least three phosphate groups and a free 3′-OH end act as substrates. Terminal transferase can be used for 3′-homopolymer tailing and accepts not only the naturally occurring deoxynucleoside triphosphates but also triphosphates with modified bases and biotin-linked bases.[280] The cofactor required for purine bases is Mg^{2+}, and that for pyrimidine bases is Co^{2+}.[281] 3′-Radioactive labeling using ^{32}P-labeled 3′-deoxynucleosides[282] and 2′-deoxyribonucleosides[283] is also possible.

III. Properties of the Antisense Oligodeoxynucleotides and Resultant Problems

For the antisense oligonucleotide principle to be put into practice the nucleic acid derivatives employed must comply with the following requirements:[284-287] (1) The complex formed between the oligonucleotide and its complementary target sequence must be sufficiently stable under physiological conditions. (2) The interaction between the oligonucleotide and its target sequence must be specific. The specificity is determined by the defined base sequence and the resulting interaction with the complementary nucleic acids. (3) The oligonucleotide must have a sufficiently long half-life under in vivo conditions for it to be able to display its desired action in the cell. It must therefore be resistant to enzymes that degrade nucleic acids (nucleases). (4) The oligonucleotide must be able to pass through the cell membranes to reach its site of action. Uniform distribution of the oligonucleotide will normally be desirable; i.e., it should not accumulate either in particular organs such as the liver or in particular cell compartments such as the lysosomes.

Whereas the requirements for specificity and binding affinity are satisfactorily met by the unmodified oligonucleotides, adequate stability to nucleases and sufficient passage through membranes can be achieved only by modification of the oligonucleotides. However, the derivatization often has an adverse effect on specificity and binding affinity. Thus, the development of antisense oligonucleotides involves walking a tightrope, with all four basic requirements needing to be met at all times.

A. Physicochemical Properties

1. The T_M Value

The physicochemical properties are one of the major determinants of the efficiency of antisense oligonucleotides. The affinity of the binding between the oligonucleotide and its target sequence is characterized by the melting temperature T_M of the double-stranded

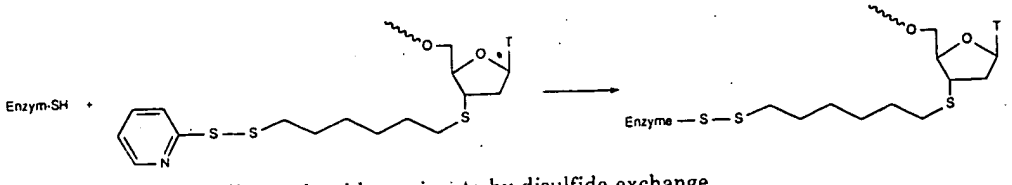


Figure 41. Synthesis of an enzyme–oligonucleotide conjugate by disulfide exchange.

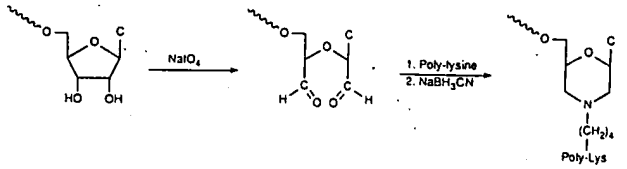


Figure 42. Synthesis of a 3′-polylysine conjugate.

nucleic acid that is formed. T_M is the temperature at which 50% of the double strand has dissociated into its two single strands. T_M depends on the concentration of the oligonucleotide and on the properties of the solvent. To estimate T_M for unmodified oligonucleotides with a length of ca. 12–20 bases the rule of Wallace[26,288] can be used, which holds true for double-stranded DNA with perfect base pairing at high salt concentration

$$T_M = n(2\ °C) + m(4\ °C) \qquad (1)$$

where n = number of dA·T base pairs and m = number of dG·dC base pairs.

The stability increases with the proportion of dG·dC base pairs as a result of the three hydrogen bonds formed by this pair. Thus, under physiological conditions (37 °C, low salt concentration), at least 12 base pairs are required to achieve reasonably stable hybridization of the oligonucleotide with the target sequence. A somewhat more accurate prediction of T_M is obtained with the free energy parameters and using the nearest-neighbor model,[289] the accuracy being about 5 °C for oligonucleotides. In practice, a 20-mer oligonucleotide with average base pairing may be assumed to have a T_M of 54 °C (DNA duplex in 0.1 M NaCl).[290] The model of Damle[291] can be used to calculate from T_M the thermodynamic parameters δH and δS for the binding of an oligonucleotide to polynucleotides, as follows

$$1/T_M = \delta S/\delta H + 2.3R/\delta H \log C_M \qquad (2)$$

where C_M = concentration of free oligonucleotide at $T = T_M$.

Watson–Crick base pairing permits the formation of double strands between DNA and DNA, DNA and RNA, and RNA and RNA, with the stability of the double strand decreasing in the sequence RNA·RNA > RNA·DNA > DNA·DNA.[224] The extent of double-strand formation can be checked by gel migration analysis[292] because the double-stranded molecule moves different from the corresponding single strands on polyacrylamide gel electrophoresis.

2. Effect of the Internucleotide Phosphate Modification on the Binding Affinity

Modification of an oligonucleotide may result in an increase or decrease in T_M. If the internucleotide group is altered by introduction of a new substituent into the phosphate center, the factors that determine the T_M are (1) the electronic nature (charge), (2) the steric requirement, and (3) the absolute stereochemical arrangement of the substituent. The charge repulsion will be less with uncharged internucleotide linkages (phosphate esters, methylphosphonates, and dephospho compounds) than for the natural phosphodiesters and thus the duplex should be stabilized. There has been some experimental confirmation of this,[292–294] but there are discrepancies with some other investigations.[134,295,296] There are various reasons for this. First T_M depends on the ionic strength of the buffer used.[292] It is an important point that this dependency of the T_M on the ionic strength is found only with the phosphodiester compounds and not with the phosphotriester analogues.[294] Second, the stoichiometry of the hybridization is often different from 1:1 so that the hybrid molecules undergoing dissociation are not always equivalent. Sarin et al. have also reported that the melting curve for oligonucleotide methylphosphonates was broadened when they had been insufficiently purified.[295] The stability of the complexes formed from the nonionic homooligomeric dA methylphosphonates with poly(rU) was greater than that of the corresponding natural phosphodiesters.[294] The stoichiometry of complex formation in this case is 2rU·1dA and 2T·1dA. By contrast, Tidd et al. found a 1:1 complex on sequence-specific hybridization of a 9-mer methylphosphonate with a 20-mer oligodeoxyribonucleotide.[296] However, only a small proportion (10–20%) of the stereoisomers was at all capable of producing this hybrid population with a T_M of 34 °C. Most of the stereoisomers were in dissociated form at 25 °C. The depression of the T_M values was less pronounced in an investigation by Sarin et al. on 20-mer methylphosphonates.[295] Whereas the unmodified 20-mer had a T_M of 55 °C, this was reduced to 53 °C for the same sequence with four phosphonate residues and to 51 °C with 18 phosphonate residues.

When Stein et al. compared the T_M values for phosphorothioates with the unmodified oligonucleotides, they made the interesting observation that A·T pairs depress the T_M values considerably more than do G·C base pairs.[235,297] The position of the phosphorothioate residue in a partially modified oligonucleotide also appears to influence the T_M. A phosphorothioate in the 5′ position in front of a pyrimidine residue lowers T_M more than when in front of a purine residue.[298,332]

The fact that the binding affinity of an antisense oligonucleotide crucially determines its efficiency in a biological assay is evident from the fall in biological activity as the assay temperature increases. Modified antisense oligonucleotides are often more active at 25 °C than at 37 °C.[299–302] The observation that the activity of oligonucleotides increases with their chain length is in the same direction.[290,303–305] This correlation disappears above a particular chain length, which is about 30 bases for natural oligonucleotides. The explanation for this is obvious because the T_M of large natural DNA molecules ranges from 80 to 95 °C, irrespective of their particular length, although it depends on the G·C content, which, in turn, is species dependent.

3. Approaches To Improve the Binding Affinity

Hélène et al. covalently linked intercalating agents to the oligonucleotide with the aim of increasing T_M. Attachment of an acridine residue to the 3′ end of $(T)_{12}$ increased T_M from 33.5 to 47.3 °C.[261] The length of the alkyl chain connecting the acridine to the antisense oligonucleotide had a crucial effect on duplex stability. Another possibility for increasing T_M comprises introduction of modified bases that form more stable Watson–Crick base pairs with the complementary bases owing to additional hydrogen bonds. For example, diaminopurine (DAPu) forms three hydrogen bonds

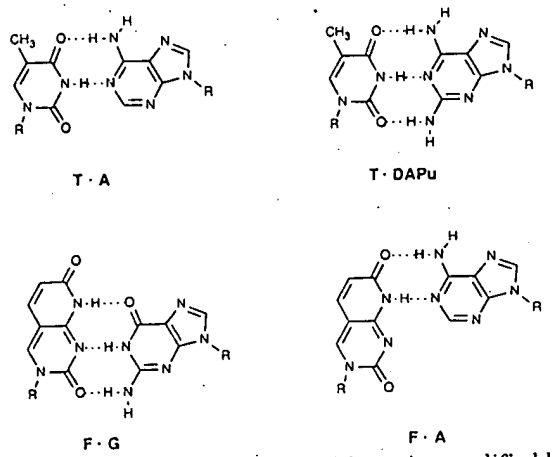


Figure 43. Enhancement of base pairing using modified bases (DAPu = diaminopurine, F = pyridopyrimidine).

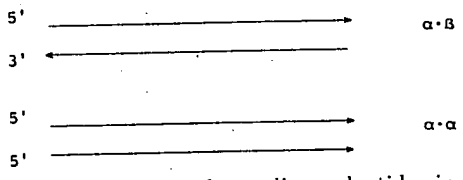


Figure 44. Hybridization of two oligonucleotides in the antiparallel orientation as α·β duplex (top) and in the parallel orientation as α·α duplex (bottom).

with thymine, whereas the natural partner adenine is able to form only two hydrogen bonds.[201] The pyridopyrimidine bases (F) publicized by Inoue et al. similarly pair with guanine more strongly than does natural cytosine and thus stabilize the duplex[204] (Figure 43).

Whereas introduction of a phosphate residue at the 3′ end of the antisense oligonucleotide has no noticeable effect on T_M, the introduction at the 5′ end markedly stabilizes the double helix.[306] The improved base pairing may also be the reason why cleavage of the 5′-phosphates of the duplexes of d(GGAATTCC) with *EcoRI* is about 8 times, and of d(GGTTAACC) with *HpaI* is 30 times, faster compared to that of the corresponding 5′-hydroxy derivatives.[96,307] In this context it is surprising that attachment of relatively large molecules, for example, nucleases, to the 3′ end of oligonucleotides only slightly lowers their T_M values.[308] It is also possible, interestingly, to raise the T_M values by altering the sugar. For example, Inoue et al. have reported a 9-mer 2′-deoxyoligonucleotide to have on hybridization with a complementary oligoribonucleotide a T_M of 41 °C. As an oligoribonucleotide or a 2′-*O*-methyloligoribonucleotide the same sequence shows a T_M of 50 or 54.3 °C, respectively.[224] A drastic positive change in T_M was found by Imbach et al. for oligonucleotides that have the α-anomeric nucleosides incorporated in place of the natural β-anomers.[182,183] For example, a α-d($G_2T_{12}G_2$)·RNA duplex melts at 53 °C, while the corresponding β-anomeric duplex melts at only 27 °C. This surprising effect was explained, on the basis of investigations by NMR spectroscopy, by proposing that hybridization of α-oligonucleotides with β-DNA results in a parallel arrangement of the strands, whereas the natural double helix consists of antiparallel strands.[184,185] (Figure 44). In this case the 2′-deoxyribose unit is in the C_3′-endo conformation (Figure 32). Remarkably, the B-type DNA is retained even when the strands are arranged in parallel, and the base pairing

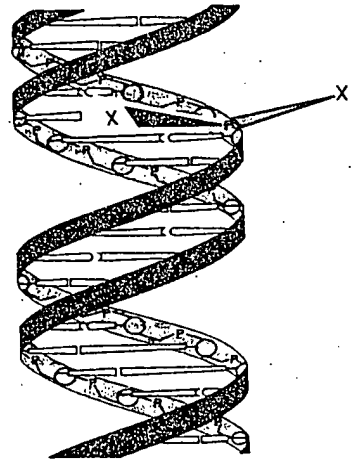


Figure 45. Assignment of the stereochemistry of a phosphate substituent in double-stranded DNA.

is consistent with a Watson–Crick model. The stabilization of the hybridization appears to be less favorable for purine-rich α-anomeric oligonucleotides than for pyrimidine-rich sequences.[309]

Some dephospho modifications lower T_M very greatly. For example, it was impossible to determine T_M for oligonucleotides with diisopropylsiloxane[152] or carbamate[166] bridges. Surprisingly, Summerton reported a dramatic increase in T_M when the carbamates are expanded to a morpholine ring[167b] (Figure 29). For p-$(dC)_6$·p$(dG)_6$, T_M increases from <30 to 64 °C when the natural DNA·DNA duplex is changed to the carbamate·DNA duplex. However, it should be pointed out that homooligomers of guanylate tend to aggregate, which means that T_M determination is no longer possible.[297]

A promising approach is that of Letsinger et al., who used cationic oligonucleotides, which ought to have an increased binding affinity for natural anionic polynucleotides.[122,310] The binding of the positively charged oligonucleotide can be controlled by the salt concentration and the pH. The binding of a cationic 10-mer deteriorates (T_M = 32.5, 27.5, 15 °C) with increasing NaCl concentration (0, 0.1, 1 M) while, conversely, that of the natural oligonucleotide improves (T_M = <10, 22, 38 °C). This effect is desirable because the ionic strength in the cell is low.

B. Stereochemical Problems

Elimination of the negative charge on the phosphate center ought, by its nature, to increase T_M. However, this does not occur in every case. With some phosphate triester oligonucleotides, which are not subject to the charge repulsion of the phosphate diester backbone, the T_M values are greatly lowered. This can be explained by stereochemical considerations. The introduction of a substituent on the phosphorus generates a new center of chirality. In principle, the substituent X that has been introduced can point into the interior of the DNA double helix or outward into the surroundings (Figure 45), and the latter is considerably more sterically favored. Bower et al. used NMR and UV spectroscopy to show, with methylphosphonate oligonucleotides, that DNA duplexes with substituents directed inward into the wide groove are generally less stable than those whose methyl groups project into the solvent.[293] At

various positions in the self-complementary oligonucleotide d(GGAATTCC) a phosphate diester linkage was replaced stereospecifically by a methylphosphonate residue. Although the R_pR_p isomeric duplexes have similar T_M values to the parent molecule, the T_M of the S_pS_p isomers was lowered markedly by ca. 7–11 °C. The steric effect of the methyl group increased with its proximity to the center of the molecule. In the case of the monophosphorothioate oligonucleotide d[GG(S)-AATTCC], LaPlanche et al. found the following sequence of T_M values by comparison with the parent molecule mentioned above: unmodified $\approx S_pS_p > R_pR_p$.[104] In the case of the ethyl phosphotriester d-[GGAA(Et)TTCC], too, the melting temperature of the R_pR_p duplex was ca. 11 °C lower than that of the S_pS_p diastereomer and the parent compound.[134]

However, the situation becomes more complicated when more than one phosphate residue is modified. Modification at n centers will result in 2^n diastereomers. At a chain length of 21 bases, corresponding to 20 internucleotide linkages, this amounts to 1 048 576 isomers. It is clear from the stereochemical effects discussed previously that not all isomers will bind equally well to the target sequence. Tidd employed affinity purification to separate a mixture of 9-mer methylphosphonates into strongly binding and weakly binding fractions.[296,311] This entailed the hybrids consisting of the 9-mer and a complementary 20-mer oligonucleotide being separated out by binding to a weak ion exchanger and the enriched 9-mer being recovered by thermal dissociation. Hybridization experiments with the two fractions revealed that the T_M of the strongly binding fraction was 5 °C higher, and that of the weakly binding fraction was 2.6 °C lower, than that of the initial mixture. Improved fractionation would be achieved in an affinity chromatography in which the complementary oligonucleotide is covalently attached to the support.

In contrast to the methylphosphonates, the chirality of the phosphorus in the methyl phosphate triesters apparently has no effect on the strength of hybridization.[312] The reason given for this is the preference of the methylphosphonates to form nonhelical conformations. For example, the melting temperature of a methyl phosphate triester 12-mer oligonucleotide is 55 °C, whereas that of a methylphosphonate 12-mer of similar sequence is only 32 °C. The T_M of an unmodified oligonucleotide is ca. 40 °C. Like the α-anomeric oligonucleotides, which likewise bind more strongly than the normal oligonucleotides, the arrangement of the strands in the hybrid molecule has also been found to be parallel for the hexathymidine pentaphosphate pentamethyl ester.[313] This hexamer hybridizes with itself via T·T base pairings. It would be interesting in this connection to know whether the stable carbamate duplexes[167b] likewise hybridize in parallel orientation.

C. Specificity

1. Specific Effects

One of the basic requirements to be met by antisense oligonucleotides is absolutely specific binding to the target nucleic acid, whose base sequence must be known. Statistically, the sequence of a 17-mer oligonucleotide occurs just once in the human genome. When it is remembered that not all the genes in the cell are switched on at the same time, but that only about 10–20% of the genes are being transcribed into the corresponding mRNA at a particular point in time, it is clear that oligonucleotides with 15–20 bases offer the opportunity of extremely selective intervention in gene expression. This high specificity and the universality are the reasons for the attractive advantages of the antisense oligonucleotide strategy. However, in order to meet the requirements for stability and passage through membranes, the structure of the intrinsically highly specific natural DNA must be modified. This may have an adverse effect, and in rarer cases a positive effect, on the specificity of the antisense oligonucleotides.

An instructive example of the high specificity of unmodified antisense oligonucleotides is found in the study by Holt et al.[314] about the part played by the nuclear protooncogene *c-myc* in the regulation of cell growth and differentiation. The antisense oligonucleotide directed against the *c-myc* mRNA contained 15 bases. A search for homologous sequences in the GenBank databank revealed a number of potential target sequences with up to 13 bases identical with the total of 15. Remarkably, the 15-mer antisense oligonucleotide was able to inhibit specifically the proliferation of HL-60 cells employed and to induce differentiation. Related oligonucleotides of the same length but with 2–12 mispaired bases were unable to influence the growth of HL-60 cells. Comparably high specificities were found by Anfossi et al. in studies of *c-myb* protooncogene expression.[315] The experimental procedure for examining the specific action of the antisense oligonucleotides was as follows: the oligonucleotide that is complementary to the antisense oligonucleotide, which is also called the sense oligonucleotide, did not inhibit gene expression, in contrast to the antisense oligonucleotide.[316] However, an excess of the sense oligonucleotide was able to abolish the action of the antisense oligonucleotide. This rules out a general toxic effect of the antisense oligonucleotide as the principle of action. In the inhibition of β-globin synthesis by antisense oligonucleotides, Goodchild et al. found specificity for the β type whereas earlier experiments had also shown nonspecific inhibition of α-globin synthesis.[290] In addition, a 19-mer antisense oligonucleotide brought about specific suppression (100%) of the expression of a functional *Torpedo* acetylcholine receptor in the oocyte test. By contrast, the expression of the homologous cat muscle acetylcholine receptor was inhibited by only 47%.[317] This finding also indicates that there is no nonspecific toxic effect by the antisense oligonucleotide.

2. Nonspecific Effects

Nonspecific effects of antisense oligonucleotides have also been reported. Unmodified oligonucleotides appear to cause, when injected into *Xenopus* embryos, a nonspecific breakdown of the mRNA at the injection sites.[318] Marked specificity problems have been found with the phosphorothioates. Zamecnik et al. investigated phosphorothioate antisense oligonucleotides for their antiviral activity against HIV (human immunodeficiency virus).[102] At an oligonucleotide concentration of 4 μg/mL they found an effect only with those antisense oligonucleotides complementary to the viral RNA.

However, at the higher dose of 20 μg/mL, a phosphorothioate oligonucleotide that had no binding site on the viral RNA had an effect similar to that of the oligonucleotide with the designed binding site. Hence it appears that the phosphorothioate oligonucleotides can act by different mechanisms depending on the used doses (cf. section IV.D). It is even more surprising that homooligomeric phosphorothioate oligonucleotides are likewise active against HIV[319] with the effectiveness of a 14-mer homooligonucleotide decreasing in the sequence dC > T > dA. Corresponding phosphorothioates of the 2′-*O*-methyloligoribonucleotides showed a similar lack of specificity in the HIV test, in which the inosine derivative was more effective than the cytidine derivative.[226]

The antiviral activity of the methylphosphonate oligonucleotides is less than that of the phosphorothioates, but the action of the former is generally more specific. An 8-mer methylphosphonate antisense oligonucleotide that had one phosphodiester linkage and was complementary to the splice acceptor site of HSV-1 (herpes simplex virus type 1) had a sequence-specific anti-HSV action, whereas a similar oligonucleotide without a viral binding site had no antiviral activity.[320] In another experiment by Miller et al., methylphosphonate antisense oligonucleotides against the mRNA of VSV (vesicular stomatitis virus) were tested for inhibition of viral protein biosynthesis.[321] They synthesized three different methylphosphonate oligonucleotides against the initiation regions of mRNA of the N, NS, and G proteins of VSV. The N oligonucleotide specifically inhibited N protein synthesis. By contrast, the NS oligonucleotide suppressed the synthesis of both the NS protein and the N protein. The diminished specificity was attributed to the formation of partial duplexes between the NS oligonucleotide and the mRNA for the N protein. When it is remembered that the oligonucleotides used were only 9 bases long, this explanation seems somewhat unsatisfactory. A methylphosphonate oligothymidylate that was also tested as a control exerted only a weak inhibitory effect on viral protein synthesis.[247,321]

With regard to the specificity of antisense oligonucleotides that contain a bound intercalating agent such as acridine, it may be thought initially that there will be a reduction, because aminoacridines intercalate as such into DNA and, as part structures in other derivatives, have enzyme-inhibitory properties.[322] However, the inhibition of influenza virus investigated by Hélène et al. was found to be highly specific.[262] A heptanucleotide with a 3′-terminal acridine unit selectively suppressed the cytopathic effect of influenza virus type A, whereas it had no effect on type B viruses. The target sequence was the 3′ terminus of the type A virus, which differs in four of twelve bases from the type B virus. This high specificity of the intercalating oligonucleotides was confirmed in another experiment in which growth of *Trypanosoma brucei* parasites was specifically prevented.[236] However, in another study by the same group on some intercalating antisense oligonucleotides there were stated to be nonspecific effects on transcription.[260] The observed side effect was ascribed to the binding of the antisense oligonucleotides to RNA polymerase. This suggests a parallel to the inhibition of reverse transcriptase by nonnucleotidic acridine-containing derivatives.[322]

3. Control of Specific Hybridization

The specificity of the corresponding antisense oligonucleotides can be controlled, within limits, by altering the heterocyclic bases. Thus, the hybridization of oligonucleotides containing diaminopurine nucleosides appears, at least at high salt concentrations, to be more specific than that of normal adenine-containing oligonucleotides.[201] By contrast, incorporation of inosine reduces the specificity. Inosine pairs with all four natural bases in the sequence dC > dA > T ≈ dG.[194] As specified, a reduction in specificity appears undesirable, but it may be an advantage, for example, when the intention is to direct only one antisense oligonucleotide against a family of very similar target sequences (e.g., a family of parasites[323]) that are homologous apart from only a few positions in the base sequence.

There are some simple experimental modalities for checking the specific hybridization of antisense oligonucleotides to their target sequences in vitro. If the oligonucleotides have a free 3′-hydroxyl group that can act as a primer for DNA polymerases, sequencing by the Sanger method[324] is possible. This primer function, for which methylphosphonates and phosphorothioates are also suitable, can be used to check the correctness of the binding site.[325] Nonspecificity can be estimated qualitatively from the sequencing quality that can be achieved. Another possibility comprises examination of the protein products if the antisense oligonucleotides bind in the coding region of the mRNA. The translation stop brought about by hybridization can be used to prepare specifically shortened proteins whose size can be determined by gel electrophoresis.[304] Finally, there is also the possibility of specific cleavage of the mRNA on the points on the RNA·DNA double strand using RNase H. However, this type of cleavage is not possible with some modified oligonucleotides (cf. section IV.E).

D. Stability to Nucleases

1. Stability of Unmodified Oligonucleotides

Another major requirement to be met by antisense oligonucleotides is their stability both under assay conditions and in vivo following therapeutic use. It has long been known that natural nucleic acids are subject to catabolism in the serum and in cells. For example, the stability of an mRNA in the cell is a crucial factor determining the extent of expression in the relevant protein. Even oligonucleotides that occur endogenously in eucaryotic cells are subject to rapid breakdown.[2] The enzymes responsible for the degradation of nucleic acids (DNA, RNA), the nucleases (DNases, RNases), differ in their specificities. The most important references are nucleases specific for double strands and single strands—exo- and endonucleases—and the highly specific restriction endonucleases. The nucleases that degrade single strands, especially the exonucleases, are important in the context of antisense oligonucleotides: unmodified oligonucleotides are degraded within a few hours in calf serum. Under optimal conditions the enzymatic cleavage may be complete after only 15 min.[326] On examination of the breakdown of a 5′-labeled oligonucleotide (20-mer) it becomes evident that the nu-

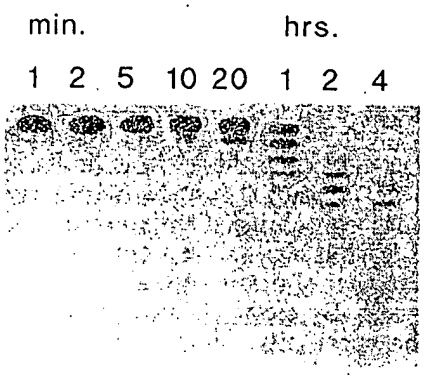


Figure 46. Kinetics of exonucleolytic cleavage of a radiolabeled 20-mer oligonucleotide in fetal calf serum (12% polyacrylamide gel).

cleolytic cleavage in serum is mainly brought about by exonucleases (Figure 46). This is indicated by the ladder-like breakdown pattern, because cleavage by endonucleases would give rise to smaller fragments even after a shorter incubation time. We were able to show by further investigations on oligonucleotides whose 5′ and 3′ ends were each protected against nucleases that a 3′-exonuclease activity is responsible for the degradation of oligonucleotides in calf serum. This 3′-exonuclease activity corresponds to that of snake venom phosphodiesterase, which degrades oligonucleotides with a free 3′-hydroxyl group from the 3′ end. Oligonucleotides whose 5′ end has been protected by modification are not substrates of spleen phosphodiesterase. These findings are consistent with the results obtained by other groups on variously modified oligonucleotides.[191,314,326,327]

To improve the cell culture test on unmodified oligonucleotides, the nucleases in the serum that is used are commonly inactivated by heat treatment. However, care is necessary with this because some batches of fetal calf serum apparently contain heat-resistant nucleases, which may cause the results to be wrongly interpreted. In addition, the rate of oligonucleotide degradation in each cell culture depends on the cell type used. Whereas a 20-mer oligonucleotide suffers extensive breakdown within 3 h in HeLa cells, it is virtually stable overnight in a culture of chicken embryo fibroblasts.[328] The half-life of a 5′-phosphorylated oligonucleotide (15-mer) in a culture of HL-60 leukemia cells using a heat-inactivated calf serum was 1–2 days.[314] Wickstrom has reported a systematic study on the stability of oligonucleotides on various subcellular extracts and culture media.[326] Oligonucleotides are virtually stable for 2 h in rabbit reticulocyte lysate or Dulbecco's modified medium containing 5% fetal calf serum. By contrast, they are completely degraded within 2 h in a postmitochondrial cytoplasmic HeLa cell extract and in 15 min in bovine calf serum. The different results with reticulocyte lysate and with HeLa cell lysate suggest that breakdown depends on the cell compartment. There is rapid degradation of oligonucleotides in some cell compartments, such as the liposomes, while it takes place distinctly more slowly in other parts of the cell, for example in the cytoplasm.[326] Unmodified oligonucleotides are relatively stable inside human T lymphocyte cells, too.[316] In summary, it can be said that oligonucleotides with a natural phosphodiester linkage are unsuitable for in vivo studies and need to be stabilized to nucleases by modification.

2. Stabilization of Oligonucleotides to Nucleolytic Degradation

An obvious approach to stabilization is to modify the phosphate center, which is where the nucleolytic attack occurs. An apparently slight change is to replace an O atom by an S atom. De Clercq and Eckstein described phosphorothioate polynucleotides with increased resistance to nucleolytic breakdown long ago.[87] In a number of publications, Eckstein has reported phosphorothioate analogues of nucleotides as tools for investigating biochemical processes and the practical use of this class of compounds.[86,329–331,333] Phosphorothioates have also been used for studies on RNA[334,335] and 2′-5′-linked oligoadenylates.[336–339]

Phosphorothioate antisense oligonucleotides are stable to snake venom phosphodiesterase and spleen phosphodiesterase, which are exonucleases.[102,319] The half-life of unmodified oligonucleotides under HIV assay conditions is 17 h, whereas no significant breakdown of phosphorothioate oligonucleotides was found after 1 week.[319] The cleavage of phoshorothioate-containing DNA duplexes by specific endonucleases such as *Eco RI* is usually stereospecific, with one diastereomer being cleaved slowly and the other usually being completely resistant.[97a,130] The phosphorothioate groups of both diastereomers of d[AA(S)AA] are resistant to staphylococcal nuclease, DNase I, and DNase II.[333]

The nonionic methylphosphonate oligonucleotides were used as antisense oligonucleotides for the first time by Miller and Ts'O.[76] In the case of dithymidylate whose internucleotide linkage was in the form of methyl- or phenylphosphonate, it was found that both compounds are stable to spleen phosphodiesterase but one diastereomer in each case is slowly hydrolyzed by snake venom phosphodiesterase.[62] Methylphosphonates are stable to endonucleases such as S1. Oligonucleotides whose backbone is constructed of alternate methylphosphonate and phosphate diester internucleotide linkages are stable to spleen phosphodiesterase and S1 but are slowly hydrolyzed at the phosphodiester linkage by snake venom phosphodiesterase.[340]

A third class of phosphate-modified oligonucleotides comprises the phosphoramidates, which, like the phosphorothioates, are stable to snake venom phosphodiesterase, spleen phosphodiesterase, and S1 endonuclease.[102,341] A fourth class is found in the phosphate triesters, which have been little used to date as antisense oligonucleotides, possibly because of their potential alkylating properties. Isopropyl phosphate triesters are, as expected, not cleaved by endonucleases.[128] Interestingly, ethyl phosphate triesters are deethylated after being taken up by the cell and then undergo nucleolytic breakdown.[76,342] However, phosphate triester oligonucleotides appear to have a mutagenic potential.[343]

The oligonucleotides that have been changed to the α-anomers in the sugar moiety are very stable to both endo- and exonucleases. Slow cleavage is found only on incubation with snake venom phosphodiesterase.[188] The stability in *Xenopus* oocytes of the α-anomeric oligonucleotides ($t_{1/2}$ > 8 h) is considerably better than that of the β-anomers ($t_{1/2}$ = 10 min).[191] According to Sproat[227] the 2′-*O*-methyloligoribonucleotides are completely resistant to RNA- and DNA-specific nucleases,

but are cleaved with varying efficiency by the less specific RNA/DNA-recognizing nucleases. Compared with DNA, the rate of hydrolysis of 2′-*O*-methyloligoribonucleotides is ca. 100 times slower with micrococcal nuclease, ca. 10 times slower with P1 nuclease, and about the same with snake venom phosphodiesterase. The partial instability to nucleases makes additional stabilization of 2′-*O*-methyloligoribonucleotides necessary, for example, as described, as combination with phosphorothioate residues.[226] Open-ring sugar analogues of adenosine have been found by Hakimelahi et al. to be stable to snake venom phosphodiesterase and spleen phosphodiesterase.[344] However, it is unclear how far such drastic changes in the molecule still allow good hybridization with mRNA. Cyclic oligonucleotides whose synthesis has been described by van Boom[345] had to be protected from endonucleases although they were stable to exonucleases.

The same stabilization toward exonucleases can be achieved by incorporating two successive blocked internucleotide linkages. Stec et al. have reported oligonucleotides that have two successive phosphorothioate residues and are not cleaved by snake venom phosphodiesterase.[97a] A 15-mer oligonucleotide protected similarly by 3′,5′-terminal phosphorothioates has a half-life of more than 1 month in 50% human serum, compared with 2–3 days for the normal 15-mer. This means that the terminally modified oligonucleotide is almost as stable as the corresponding all-phosphorothioate in the serum.[297] A third phosphorothioate internucleotide linkage, however, appears to confer no further stabilization to exonucleases.[97b] Shibara et al. reported a 20-mer 2′-*O*-methyloligoribonucleotide in which five internucleotide linkages at the 5′ end and three at the 3′ end were in the form of phosphorothioate and which was almost as active as a corresponding all-phosphorothioate against HIV-1 in vitro.[226] They attributed this effect to an increased resistance to exonucleases, because the corresponding oligonucleotide with phosphodiester linkages did not inhibit growth of HIV. However, the situation in vivo is more complicated because of the presence of endonucleases. For example, in *Xenopus* embryos breakdown of normal oligonucleotides, terminally protected oligonucleotides, and all-phosphorothioates is relatively rapid, with $t_{1/2}$ < 30 min.[346] By contrast, in *Xenopus* oocytes the phosphorothioates are considerably more stable ($t_{1/2}$ > 3 h) than the oligonucleotides without or with only partial sulfur modification.

Oligonucleotides with terminal modification by two adjacent methylphosphonate groups are much more stable to nucleases than are those with only one methylphosphonate group.[70,252,295] The explanation of this is that snake venom phosphodiesterase can jump over an internucleotide bridge as it were and liberates a dinucleotide methylphosphonate.[252] An observation that can be interpreted in the same way has also been made with phosphate triesters.[130]

Of course, many other derivatizations at the 3′ or 5′ end of oligonucleotides also protect against nucleolytic breakdown, examples being intercalating agents,[193,235,236,259] polylysine,[255,347] or poly(rA).[348] Even a single 5′-phosphate residue protects an oligonucleotide against breakdown by spleen phosphorodiesterase[349,350] because this enzyme requires a free 5′-hydroxyl group on the oligonucleotide to cleave it. In vivo, oligonucleotides of this type are, of course, instantly dephosphorylated by phosphates and then degraded. Finally, mention may be made of the dephosphooligonucleotides such as carbamates,[165] siloxanes,[151,152] or "plastic" DNA,[351] which are completely nuclease resistant but which have unsatisfactory hybridization and solubility properties.

E. Penetration through Membranes

1. Mechanism of Cellular Uptake of Oligonucleotides

It is essential for the activity of a therapeutic of any type whatever that its bioavailability is satisfactory. Thus, the activity of the antisense oligonucleotides is crucially affected by how well they reach their site of action unmetabolized. The protein biosynthesis apparatus of the cell is located in the cytoplasm and comprises 55% of the cell volume. Thousands of enzymes bring about there the biosynthesis of sugars, fatty acids, nucleotides, amino acids, and proteins. The mRNA produced in the nucleus by transcription of the DNA is translated into the corresponding protein on the ribosomes in the cytoplasm. In order for the antisense oligonucleotides to be able to act to stop translation by hybridization, they must pass through the plasma membrane into the interior of the cell. The plasma membrane is a natural barrier to many large or negatively charged molecules. It might therefore be supposed that this membrane barrier would form a bottleneck in the antisense oligonucleotide concept.

Surprisingly, however, cellular uptake of the oligonucleotides takes place better than would have been expected from a polyanionic compound of this size. The uptake of radiolabeled oligonucleotides takes 15 min to a few hours depending on the type of cells used and the experimental conditions. Goodchild reported that the process of uptake is energy dependent because it is inhibited by dinitrophenol, an inhibitor of ATP synthesis.[328] The active transport of the oligonucleotides takes place by endocytosis, which, according to all the evidence, is receptor mediated.[352] Affinity chromatography on oligo(T) cellulose was used to isolate an 80-kDa surface protein that might be responsible for oligonucleotide transport. The membrane receptor hypothesis is supported by the finding that phosphorothioate oligonucleotides, but not methylphosphonate oligonucleotides, inhibit the uptake of fluorescence-labeled oligonucleotides with normal phosphodiester linkages.[235] However, the introduction of a fluorescence label may also change the penetration characteristics of oligonucleotides. Also worthy of note is the receptor-mediated uptake of the DNA of bacteria phage *lambda* in white blood cells, which possibly presents a general mechanism of uptake of exogenous DNA.[353]

However, there is also the possibility, especially for modified oligonucleotides, of nonspecific binding to the membrane. The membrane-bound oligonucleotides might then gradually be internalized by the process of membrane reorientation.[354] Passive uptake into the cell appears to be confirmed for methylphosphonate oligonucleotides.[76,126,301] It appears necessary, and not least for this reason, to define the term "cellular uptake". In his early work between 1971 and 1974 on the uptake of polynucleotides by animal cells, Schell left two possibilities open.[355,356] According to his definition, cellular

uptake comprises both penetration of the polynucleotides into the interior of the cell and their irreversible binding to the cell surface. Zamecnik et al. were able, however, by electroporation experiments and investigations with the electron microscope to demonstrate clearly that the oligonucleotides are located inside the cell.[252]

It has been estimated that in cell culture in the micromolar concentration range the intracellular concentration of oligonucleotide is ca. 7.5–10% of the concentration outside.[327,328] Studies of the penetration of tritium-labeled oligonucleotide to HeLa cells showed that the radioactivity was mainly on the outside of the cells after a very short incubation time, whereas the label was located exclusively in the nucleus after 15 min.[328] Although only 6% of the oligonucleotides were broken down to free nucleosides outside the cell, it is difficult to say how much of the intracellular radioactivity is attributable to the free nucleoside. Investigations on nuclear hsp70 transcripts[357] and on antisense oligonucleotides against the splice sites of HSV and HIV[320,327] also support the possibility of penetration as far as the nucleus, because both transcription and splicing of the RNA take place in the nucleus (cf. section IV.C). The time course of the cellular uptake of oligonucleotides is linear in the initial phase and then reaches a plateau that usually lasts from 2 to 3 h.[316] With acridine-labeled oligonucleotides in HL-60 cells, this plateau phase is not reached until after 50 h. The concentration that can be reached inside the cell is inversely proportional to the length of the oligothymidylate used in the range from 3 to 20 bases.[352]

The cellular uptake of methylphosphonates is also time dependent in a similar manner.[126] At least the small methylphosphonate oligonucleotides appear to be taken up very efficiently by animal cells, and no dependence on the chain length was found in the range from two to nine bases.[76] Interestingly, *E. coli* B cells do not take up any methylphosphonate oligonucleotides with more than four bases, whereas *E. coli* ML 308-225 displayed no problems with the cell uptake of these oligonucleotide derivatives.[301] It has been found that the trinucleotide ethyl phosphate triesters are very effectively internalized by hamster fibroblasts, but once inside the cell they are deethylated and metabolized.[342] Surprisingly, phosphorothioate oligonucleotides do not penetrate as well as unmodified oligonucleotides. Stein et al. used flow cytometry to show that uptake of a 5′-acridine-labeled 20-mer homothymidylate phosphodiester was more efficient that that of a similarly labeled 7-mer phosphorothioate homothymidylate.[235] This process of uptake appears to be energy dependent because there is no uptake in dead cells. Moreover, uptake of oligonucleotide is, in contrast to that of free acridine, temperature dependent.

3′-Derivatization of oligonucleotides appears to have a beneficial effect on both the ability to penetrate and the stability to nuclease. In addition, when an acridine label is used it provides a simple detection method for following the cellular uptake of such oligonucleotides. Thus, the uptake of a 9-mer antisense oligonucleotide into live *Trypanosoma brucei* parasites can be detected in <2 h by means of the green fluorescence.[236]

With regard to the interpretation of individual results, it appears important to mention the coupling between penetration and breakdown by nucleases. The rate of degradation of an oligonucleotide depends on its location in the various compartments in the cell.[326] However, its location is determined by its penetration behavior, and the latter in turn is determined by the type of modification of the oligonucleotide. It is not experimentally straightforward to investigate the distribution of the oligonucleotides in various cell compartments, such as the cytoplasm, endoplasmic reticulum, mitochondria, Golgi apparatus, lysosomes, or nucleus, and the breakdown in these compartments, but it appears to be extremely important. For example, it would be of little benefit if antisense oligonucleotides were endocytosed in the cells and then mainly broken down or stored packed in lysosomes. This would mean that, despite a high intracellular concentration, they would be enveloped in a membrane and not available for inhibiting protein biosynthesis. Problems of this type were recognized and discussed early by Pitha in investigations on "plastic" nucleic acids.[171,351,358,359] Moreover, distribution of "plastic" nucleic acids in vivo is nonuniform. Thus, the nucleic acid analogues are found to accumulate mainly in the liver, spleen, thymus, and bone marrow, whereas the lungs and kidneys purge themselves of these compounds. The blood–brain barrier seems to be effective for these polymers because the concentration in the brain is near the detection limit.[171] Accumulation of "plastic" nucleic acids in particular organs may prove to be an advantage or disadvantage depending on the case. The results obtained on this class of substances are certainly not representative of antisense oligonucleotides because they have very different structures and, moreover, much higher molecular weights. Nevertheless, some of the experience collected by Pitha et al. should be taken into account in subsequent testing of antisense oligonucleotides.

Only two research groups have reported on the penetration characteristics of antisense oligonucleotides in vivo. Miller and Ts'O found after injection of methylphosphonate oligonucleotides into the tail vein of mice that there was distribution over all the organs and tissues excepting the brain.[247] Application of these methylphosphonates in the form of a cream to the HSV-infected ear of a mouse prevented lesions caused by HSV. This finding indicates that the methylphosphonate oligonucleotides are able to penetrate the mouse skin. Antisense oligonucleotides with alkylating groups also penetrate into all organs and tissues, reported by Vlassov.[253]

2. *Deliberate Improvement in the Penetration of Oligonucleotides through Membranes*

Various ways have been employed to improve the penetration of antisense oligonucleotides, the most important being incorporation in liposomes or covalent bonding to nonspecific or specific carriers. Another that may be mentioned is lipophilic modification of the oligonucleotides, but the effectiveness of this has not yet been evaluated.[252]

(*a*) *Liposomes.* Liposomes are microscopic particles composed of mono- or multilamellar lipid bilayers. They enter the cells by phagocytosis or endocytosis. Review articles deal with the use of liposomes as carriers of antimicrobial[360] and antiviral[361] products. The in-

creased antiviral activity of 2′,5′-oligoadenylates encapsulated in liposomes was reported by Lebleu in 1985.[362] Although this class of compounds operates not by the antisense oligonucleotide strategy but by activation of RNase L, it is subject to the same problems of pentration and stability to nucleases. The reported rate of homoadenylate (tetramer) incorporation in liposomes is 0.8%, resulting in an intracellular concentration of the oligonucleotide of about 20 nM. However, the actual concentration of free oligonucleotide present in the cytoplasm remains unclear. It is probable that the release of oligomer from the liposomes depends on partial breakdown of the liposomes by phospholipases or other enzymes and does not necessarily result from the low intravesicular pH.[362] We are not aware of any publications on the incorporation of unmodified antisense oligonucleotides into liposomes. However, antisense oligonucleotides with lipophilic modifications can be incorporated to the extent of ca. 50% in liposomes.[253]

One disadvantage of conventional liposomes that has hindered widespread use to date is their short half-life in serum. The cause of this is the high rate of uptake of the liposomes by the reticuloendothelial system (liver and spleen). There has recently been a description of a new generation of liposomes that still have a reticuloendothelial system/blood ratio of 0.7 after 24 h.[363] Also of very great interest are targeted *immunoliposomes*: an antibody incorporated in the liposomes ensures specific attack on those cells having the corresponding surface antigen. They have been used, inter alia, for targeting antiviral nucleosides on HSV-infected cells.[364] Furthermore, virus-infected cells sometimes appear to alter their surfaces in such a way that selective fusion with liposomes becomes possible.[365]

(*b*) *Poly-L-lysine.* A systematic investigation of the cellular uptake of polynucleotides has been published by Schell.[355,356] His findings included stimulation of the uptake of homooligoribonucleotides in tumor cells by poly-L-lysine, and he suggested two mechanisms for this. On the one hand, poly-L-lysine can form a complex with the wall of the intact cell, which, because of the net positive charge, makes the latter penetrable by polyanions. On the other hand, poly-L-lysine is able to form complexes with the polynucleotides themselves, and these complexes have high affinity for the cellular membrane.[355] Polycations were suggested to have a helix-stabilizing effect by Glaser and Gabbay in 1968.[366] It is possible that the effects of poly-L-lysine go beyond those mentioned here. For example, poly-L-lysine is able, by interacting with the cellular receptor of HSV-1, to block the binding of the virus to the receptor.[367] In this connection, polylysine of molecular weight 50 000 proved to be toxic in the micromolar range.

Lebleu et al. used poly-L-lysine to investigate the antiviral activity of modified 2′,5′-oligoadenylates[368] and antisense oligonucleosides.[255,347] A 15-mer oligonucleotide or a mixture of this oligonucleotide with poly-L-lysine was not active against VSV. However, if the antisense oligonucleotide was covalently bonded via the 3′ end to poly-L-lysine, it had a specific inhibitory effect on VSV growth in cell culture.[255] The antiviral effect in the 100 nM range that was observed is impressive because most antisense oligonucleotides had previously been effective only in the micromolar range. The stabilization of the antisense oligonucleotides to 3′-exonucleases caused by covalent bonding to the poly-L-lysine is scarcely able by itself to explain the good activity of these oligonucleotides. It is probably a combination of stabilization to 3′-exonucleases and an improvement in penetration, which may be promoted by the helix-stabilizing component of the polycation.

Unfortunately, there are some problems with the use of poly-L-lysine conjugates. Poly-L-lysine has a cytotoxic effect on almost all cell lines at concentrations of 2 μM.[347] In addition, poly-L-lysine–oligonucleotide conjugates tend to aggregate, which may give rise to experimental difficulties.[369] However, the most serious point is that although poly-L-lysine conjugates have good effects on L929 cells, they confer no protection whatever against VSV infection in other cell types such as HeLa or LM fibroblasts.[347]

(*c*) *Specific Carriers.* The remaining question is the extent to which other protein conjugates able to penetrate through the membrane in a specific manner can be used to improve the passage of oligonucleotides through membranes. A possible example is a receptor-mediated uptake process as has been described for methotrexate coupled to MBSA (maleylated bovine serum albumin).[370] This conjugate is recognized by "scavenger" receptors that are mainly present on macrophages. It has therefore been proposed for the treatment of leishmaniasis. Also of interest is the finding by Vestweber and Schatz that a double- or single-stranded 24 bp DNA that is bonded by the 5′ end to a mitochondrial precursor protein is able to penetrate into mitochondria.[371] The essence of their study is the proposal that oligonucleotides should be coupled to "neutralized" bacterial toxins in order to introduce them into the cytoplasm. By neutralization this meant mutation of the toxins with the aim of eliminating the toxicity while retaining the penetrating ability. The introduction of the antisense oligonucleotides using nonpathogenic viral coat proteins represents another possible method.

IV. Mechanism of Action

Most antisense oligonucleotides to date have been designed with the aim of inhibiting translation. Only a few have been aimed at inhibiting transcription.[231,260,312,372] The mechanism of action of an antisense oligonucleotide accordingly depends on the target sequence it seeks (cf. section VI). Although the structural requirements to be met by an effective antisense oligonucleotide have not yet been clearly defined, there are concrete theories about the mechanism of action.

A. Inhibition of Translation

A possible mechanism of translation inhibition comprises the antisense oligonucleotide binding to the point on the mRNA where translation is started by the mRNA and a number of necessary initiation factors becoming bound to the ribosomes (Figure 47). In this way the RNA·DNA duplex may result, by a direct steric effect, in preventing the ribosomes and important initiation factors binding on. In the case of eucaryotic mRNA, for example, interference with the interaction between the initiation factor 4F and the mRNA is conceivable.[373,374] If the protein biosynthesis apparatus has already gone into action on the mRNA, however,

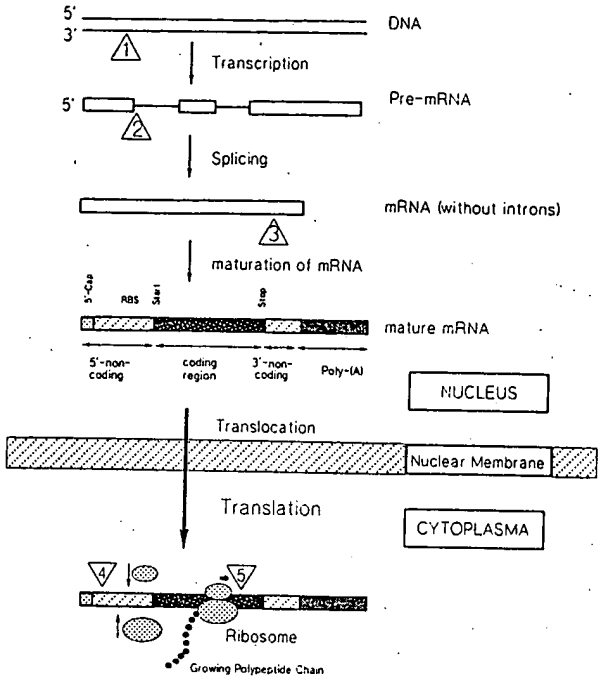


Figure 47. Mechanisms of action and target sequences of antisense oligonucleotides. [Inhibition of transcription (1), of splicing (2), of polyadenylation or translocation (3), of initiation of translation (4), or of ribosome movement along the mRNA (5).] (RBS = ribosome binding site.)

a hybridizing oligonucleotide might block the necessary translocation of the ribosomes along the mRNA. This idea has most similarity with the experiments by Dobberstein et al., who used antisense oligonucleotides against coding regions to prepare selectively shortened polypeptides of predictable length.[304] This process can be imagined to be such that the presence of an antisense oligonucleotide on the mRNA holds up the progress of the ribosomes for a time until they finally drop off. When the ribosomes dissociate from the blocked point, the growing polypeptide chains are released as polypeptidyl-tRNAs, which are subsequently hydrolyzed to the free polypeptides. All the positions investigated within the coding region were similarly effective on inhibition, irrespective of the particular reading frame. Moreover, there is only 1–2% reading over of the blockade.[304] It is not entirely clear whether, and to what extent, the ribosomes can strip the antisense oligonucleotides off the mRNA, assisted by, for example, an unwinding activity,[375] and this presumably depends on the nature of the oligonucleotide modification in the individual case. This translation inhibition due to hybridization as described above corresponds to the originally postulated mechanism action (HART).[12] However, recent findings indicate that some additional mechanisms apply, which are discussed below.

B. Inhibition of Transcription

The mRNA is produced in the cell nucleus by the enzyme RNA polymerase from the four ribonucleoside 5′-*O*-triphosphates. This entails one DNA strand acting as template. Initiation of RNA synthesis comprises the following steps: RNA polymerase recognizes specific start sequences on the DNA (promoters) and initially binds to them in the closed form (Figure 48). The closed complex is then converted into an open complex by a particular section of the double-stranded DNA

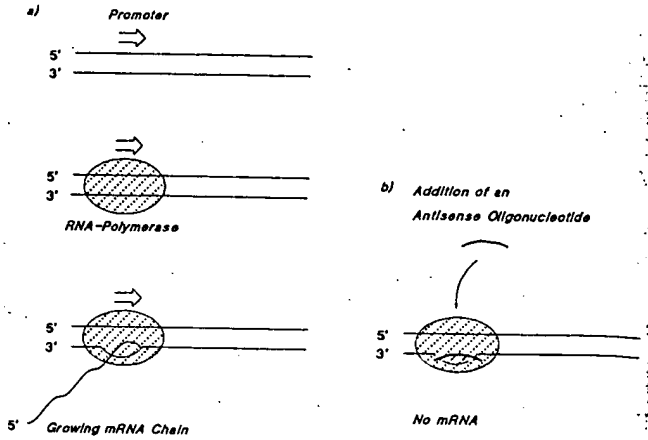


Figure 48. (a) Formation of the open promoter/RNA polymerase complex (normal transcription). (b) Inhibition of transcription by the antisense oligonucleotide binding to the open complex.

being unwound. This open complex is the stage at which the antisense oligonucleotides attack. Hélène et al. used antisense oligonucleotides having acridine derivatives bonded at the 3′ end to inhibit transcription of the β-lactamase gene.[260] The antisense oligonucleotides were hexamers to nonamers and were complementary to the transcribing strand of the β-lactamase promoter. The hexanucleotide acted specifically on the β-lactamase promoter but did not inhibit the *lac* promoter. However, both antisense oligonucleotides were found to have nonspecific effects when they were incubated with the RNA polymerase before addition of the promoter. This suggests that the oligonucleotides have nonspecific effects due to direct binding to the RNA polymerase.[231] It is worthy of note in this context that HIV reverse transcriptase is inhibited by nonnucleotidic aminoacridine derivatives.[322]

The methylphosphonate antisense oligonucleotides have been studied taking the example of blockade of transcription of the *lac* operon in *E. coli*.[312] In the noninduced state, the *lac* repressor protein binds to the *lac* operator DNA sequence and thus prevents access by RNA polymerase. Induction with isopropyl β-D-thiogalactopyranoside (IPTG) converts the *lac* repressor into a nonbinding form, and thus RNA polymerase is able to start transcription. If induction is followed by addition of an antisense oligonucleotide that is complementary to the *lac* operator sequence (repressor binding site), the result is specific inhibition of β-galactosidase synthesis in *E. coli*. It may be concluded from this that the antisense oligonucleotide has replaced the coding strand at the specified point in the DNA duplex.[312]

Hogan et al. have reported an in vitro test of the inhibition of transcription of the human *c-myc* gene by a 27-mer oligonucleotide.[372] This unmodified oligonucleotide presumably binds specifically, with the formation of a triplex structure (cf. section V.B), to a regulatory DNA sequence 115 bp upstream from the start of transcription. Astonishingly, the transcription is suppressed in the nanomolar range.

C. Inhibition of Posttranscriptional Processes

In eucaryotic cells, the primary transcript, the so-called pre-mRNA, is subject to a number of maturation processes before the mature mRNA is translocated into the cytoplasm and transcribed there. In the nucleo-

plasm, noncoding regions (introns) are cut out of the pre-mRNA (splicing), the 5′ end of the mRNA is modified to stabilize it (5′ cap structure), and various bases are altered. The polyadenylation of the mRNA at the 3′ end might be linked with the export process. Antisense oligonucleotides may intervene in any one of these processes (Figure 47).

Miller and Zamecnik showed that growth of HSV[58,320] and HIV[376] can be efficiently inhibited by oligonucleotides that bind to the splice sites of the viral precursor mRNA. In eucaryotic cells there are a few known examples of alternative splicing where two different mature mRNA species that encode different proteins can be formed from the pre-mRNA depending on the cell. The extent to which phenomena such as alternative splicing or transsplicing can be utilized, for example in a specific missplicing reaction,[252] will emerge in the future. There is already evidence that antisense oligonucleotides can prevent transport of mRNA from the nucleus to the cytoplasm: the formation of double-stranded RNA, especially in the region of the polyadenylation site,[376] prevents translocation of the mRNA into the cytoplasm.[4,190,377]

D. Non-Sequence-Specific Mechanisms

The phosphorothioate antisense oligonucleotides attracted early attention due to their exceptionally good in vitro activity against HIV in the range 0.5–1 μM.[102,284,319] However, a phosphorothioate oligonucleotide which has no binding site on the viral RNA also inhibits HIV growth at high dosage (20 μM), although there is no activity at 4 μM. This similarity of the effect of a nonspecific oligonucleotide at 20 μM to that of a specific antisense oligonucleotide indicates that there is a mechanism differing from that conceived for antisense oligonucleotides.[102] Equally surprising is the activity of homopolymeric phosphorothioates. Thus, the phosphorothioate of $(dC)_{28}$ inhibits the de novo synthesis of HIV DNA.[319] Experiments on this have shown that it is likely that the phosphorothioate $(dC)_{28}$ neither interferes with the interaction between HIV and the CD_4 receptor nor has an antiviral effect by induction of IFN-γ. De Clercq and Eckstein reported, in 1970, that the anti-VSV effect of phosphorothioate homopolymers in vitro and in vivo was greater than that of the normal phosphodiester homopolymers and was accompanied by interferon induction.[87] The inhibition of the DNA polymerase of Rauscher leukemia viruses by homopolynucleotides[378] also points to a mechanism of action based on inhibition of HIV reverse transcriptase. Only recently has the mechanism entailing direct inhibition of HIV reverse transcriptase been confirmed.[379] The phosphorothioate of $(dC)_{28}$ proved to be a competitive inhibitor of DNA synthesis with a K_i one two hundredths of that of $(dC)_{28}$. If it is assumed that the phosphorothioate reaches the cytoplasm, it is probable that inhibition of HIV growth at low concentration (<4 μM) is based on nonspecific reverse transcriptase inhibition on which is superimposed, at higher concentration (>25 μM), a mechanism based on specific hybridization.

However, the mechanism of action on HIV is intrinsically more complicated than that on normal mRNA. HIV is a retrovirus whose genetic material is RNA which is transcribed into DNA by HIV reverse transcriptase (Figure 49). Each of the steps 1–3 shown in the figure are catalyzed by HIV reverse transcriptase, which acts as an RNA-dependent DNA polymerase, an RNase H, and a DNA-dependent DNA polymerase. The stages that are shown separately for clarity in fact take place simultaneously. Mölling et al. detected the RNase H activity of HIV reverse transcriptase,[380] and it has been localized to the C-terminus of the holoenzyme.[381] The double-stranded DNA is incorporated as a provirus, with the aid of viral integrase, into the genome of the affected cells. It is evident from this that there are a large number of possible interventions which may be based on different mechanisms. A new mechanism is immediately evident from Figure 49, because viral RNase H degrades the RNA part of the RNA·DNA double strand. This is exactly the situation arising after hybridization of an antisense oligonucleotide onto the viral RNA. This means that it will be possible to attack the virus with its own weapons by using a suitable oligonucleotide to induce the viral RNase H to degrade viral RNA. In principle, the antisense oligonucleotide can be directed against a de novo infection or against chronically infected cells. De novo infections have always been investigated in the publications that have appeared so far. Matsukura et al. have now reported on phosphorothioate antisense oligonucleotides that act sequence specifically against cells chronically infected with HIV.[382] The nonspecific effect of sense oligonucleotides or homopolymers that is found on inhibition of de novo HIV infection does not occur in this case.

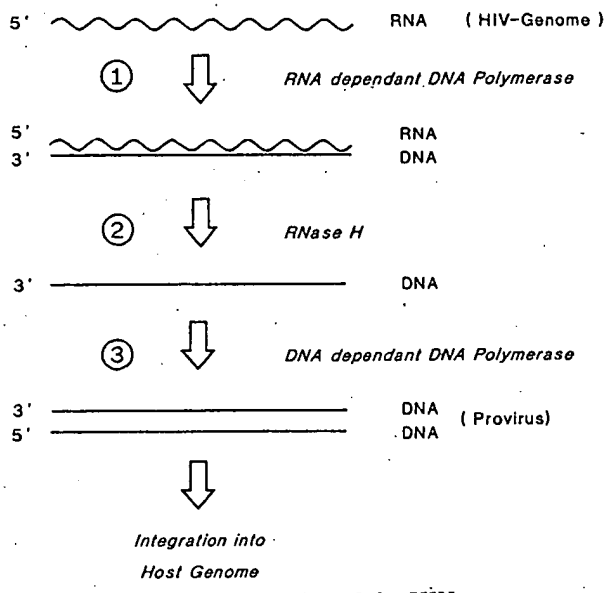


Figure 49. Reverse transcription of the HIV genome.

E. RNase H Mechanism

The excellent hybridization properties of the α-anomeric oligonucleotides were mentioned in Section III.A. However, it was sobering to find that these α-oligonucleotides are unable, despite their high binding affinity, to inhibit translation into proteins.[187] Normal β-oligonucleotides with lower melting temperatures were very effective in the rabbit reticulocyte lysate assay used. There appears to be a simple explanation for this. Hybrids of RNA and β-DNA are substrates of RNase H, but those of RNA and α-oligonucleotides are not. The inhibition of protein synthesis in these assays can

be attributed mainly to the RNase H activity.

Our attention was drawn for the first time to the possibly crucial importance of RNase H for the activity of antisense oligonucleotides by the papers of Häuptle et al.[304] and of Minshull and Hunt.[383] The latter demonstrate, using single-stranded cDNA, which is equivalent to synthetic oligonucleotides in this respect, that inhibition of translation in the cell-free wheat germ system is mainly due to RNase H. The authors noticed the following inconsistency: translation in *Xenopus* oocytes can be effectively blocked with short synthetic antisense oligonucleotides but this fails with antisense RNA transcripts, even when long. This suggested the presence of RNase H in the cytoplasm of the oocytes, because it cuts RNA·DNA but not RNA·RNA.[384] These results are consistent with the earlier work of Häuptle et al.[304] who attributed the ineffectiveness of antisense oligonucleotides in the cell-free reticulocyte assay to the RNase H activity in this lysate being too low. Cleavage with RNase H yields 5′-phosphates and 3′-hydroxyl derivatives.[385] After a lengthy controversy, the RNase H of reverse transcriptase has now also been shown to have endonuclease activity,[386] the products being mainly mono-, di-, and trimers with a 3′-hydroxyl group.[362]

RNase H occurs not only in HIV or *E. coli* but also ubiquitously in plant and animal cells. Thus, this would represent no restriction on the strategy. Since RNase H is involved in DNA replication, it is required in every dividing cell. However, although this process takes place in the cell nucleus, this does not mean that RNase H does not play a part in vivo in the antisense oligonucleotide strategy.[303] Although protein biosynthesis takes place in the cytoplasm, consideration must be given to the nuclear mechanisms discussed previously, such as inhibition of mRNA transport or of splicing or breakdown of pre-mRNA. In addition, RNase H also appears to occur in the cytoplasm.[374] As early as 1969, Stein and Hausen described an enzyme from calf thymus that cleaves the RNA part of RNA·DNA hybrids.[387]

For the conventional test systems studied, it should be remembered that although RNase H occurs in *Xenopus* oocytes and eggs, wheat germ extracts, homogenized sea urchin eggs,[388] and extracts of Krebs-2 cells,[389] it is absent from or present in insufficient quantities in the reticulocyte system.[304] The RNase H level in the test system used by various authors is often unclear. In addition, the RNase H content may vary between batches. Freshly prepared reticulocyte lysates may still contain 1–2% of the RNase H activity of live cells, which is enough for quantitative cleavage of the target mRNA.[374] Thus, the reader is often left in the dark about the extent to which the test system is subject to an RNase H dependent mechanism. An elegant procedure in this respect is that of Walder and Walder,[374] who employed a competitive inhibitor of RNase H, namely, poly(rA)·poly(T), in addition to the enzyme. This made it possible to demonstrate that those antisense oligonucleotides that hybridize entirely at the 5′ end of the mRNA exert under these conditions a significant effect, which is independent of RNase H, on translation initiation. The inhibition of translation otherwise took place by the RNase H mechanism because this does not depend on a position on the target mRNA.

Further support for this principle of action was found by the selective elimination of mRNA by RNase H in vivo. Dash et al. stated that injection of antisense oligonucleotides (15- to 30-mers) into *Xenopus* oocytes was followed by complete, sequence-specific degradation of both coinjected and exogenous mRNA.[390] The mechanism of action of antisense oligonucleotides in *Xenopus* oocytes can be regarded as taking in two stages.[357] In the first stage, RNase H cuts the mRNA in the region of DNA·RNA duplex. In the next step the cleaved fragments after their dissociation are digested by exonucleases. The degradation of internally cleaved mRNA is generally much faster than of intact mRNA because the latter is protected from exonuclease degradation by a 5′ cap or by particular structures at the 3′ end.[391,392] The only reasonable explanation of the observed high effectiveness of unmodified antisense oligonucleotides at inhibiting translation is on the basis of mRNA degradation by RNase H. This is because hybridization of the antisense oligonucleotides onto the target mRNA is a physical interaction that involves an association/dissociation equilibrium for both molecules and thus, in principle, cannot be 100% effective.

However, a major mechanism based on RNase H entails the risk of a certain lack of specificity, because even brief interactions of only 5–6 base pairs may be recognized as a DNA·RNA substrate.[357,388] This fact might also explain the nonspecific action of antisense oligonucleotides at higher concentration.[388] On the other hand, a 10-mer is the shortest antisense oligonucleotide that brings about cleavage of H4 mRNA in *Xenopus* oocytes.[318] A nonspecific mRNA breakdown may occur in *Xenopus* eggs, probably at the injection site because the local concentration here is transiently very high.

The current view is that it appears worthwhile to employ for the antisense oligonucleotide strategy those oligonucleotides whose duplex with RNA is an RNase H substrate. Methylphosphonate oligonucleotides do not meet this condition.[311,393] Although methylphosphonate oligonucleotides inhibited translation of DHFR mRNA much less well than normal oligonucleotides, this effect was not attributed to unsuitability as an RNase H substrate.[393] The reasons for this statement were the observed dependence of the inhibition of translation on the position of the binding site on the mRNA, as well as the absence of a reduction in intact DHFR-mRNA. However, the authors were unable to give a plausible explanation of the ineffectiveness of the methylphosphonates. On the other hand, Tidd's experiments with methylphosphonates support the RNase H model.[311] The absence of action of translation inhibition by the α-anomeric oligonucleotides is generally explained by the RNase H mechanism.[187,191,347] α-DNA·β-RNA hybrids are not substrates but inhibitors of the RNase H of *E. coli* or *Drosophila*.[181] By contrast, phosphorothioate antisense oligonucleotides give rise to the expected cleavage with RNase H after hybridization on RNA.[297,346,394] The substrate activity may be less[394] or more[297,394] than with unmodified oligonucleotides. For example, the RNase H activity on the duplex poly(rA)·$(T)_{40}$ is much less than on the corresponding phosphorothioate complex.[297] One possible explanation of the increased activity is provided by the lower stability of the phosphorothioate

hybrids (local melting or increased flexibility) during the cutting process. On the other hand, the cut duplex might dissociate more rapidly because of the lower stability; another alternative is a combination of the two effects. Terminally modified antisense oligonucleotides appear to behave almost like phosphodiesters,[346] with the length of the internal phosphodiester region being crucial for the suitability as a substrate. Finally, it might be conceivable that a predictable site-specific cleavage of RNA[225,395-397] could be induced by using suitably modified or chimeric oligodeoxynucleotides/oligoribonucleotides.

F. Antisense Oligonucleotides with Interactive Groups

There are in principle two different types of interactive groups on oligonucleotides. The interaction of one category of these compounds with the complementary RNA or DNA is based on chemical modifications. This includes the chemical cross-linker and photo-cross-linker oligonucleotides as well as the artificial endonucleases. The second category comprises oligonucleotides with intercalating residues that cause no change in the target nucleic acid. Category I derivatives appear particularly attractive in combination with those oligonucleotides that, after hybridization, do not allow cleavage by RNase H of the mRNA in the double strand (α-anomeric, methylphosphonate, 2′-*O*-methyloligonucleotides) and, for this reason, often have little or no activity.

1. Antisense Oligonucleotides with Intercalating Residues

The mechanism of action of antisense oligonucleotides with intercalating residues can be imagined to be such that, by reason of its special sequence, the oligonucleotide binds specifically to the target nucleic acid, with the intercalating group providing the antisense oligonucleotide with an additional binding energy which is, however, nonspecific.[261] It should be noted that the specific component is distinctly more important. The noncovalent binding of these groups is generally based on intercalation between two adjacent base pairs but may also take place by binding to the periphery of the nucleic acid. The intercalation is reversible and distorts the sugar–phosphate backbone of the double strand.

Acridine and its derivatives are the intercalating agents that have been longest known and they were also the first to be incorporated in oligonucleotides. Hélène's group reported in 1983 that duplex molecules having an aminoacridine group bonded via a phosphate residue at the 3′ end of one strand have increased stability.[258] This was followed by further investigations[189,399,400] in which the intercalating unit was bonded via the 3′ or 5′ end of normal phosphodiesters,[236,238,259,260,262,401] phosphorothioates,[235] phosphotriester/methylphosphonates,[263] or α-anomeric oligonucleotides.[237]

In general, the advantages of intercalating oligonucleotides may be said to be the following: (1) The attachment of an intercalating residue to oligonucleotides increases their binding affinity for the target nucleic acid. (2) The 3′-derivatized oligonucleotides are more stable toward 3′-exonucleases. (3) The attachment of intercalating residues usually improves the ability of the oligonucleotides to penetrate owing to increased lipophilicity. (4) The binding of acridine-modified oligonucleotides to oligoribonucleotides is much stronger than to oligodeoxyribonucleotides.[238] (5) Apart from a few exceptions,[259,260] the high specificity of the antisense oligonucleotides is retained.

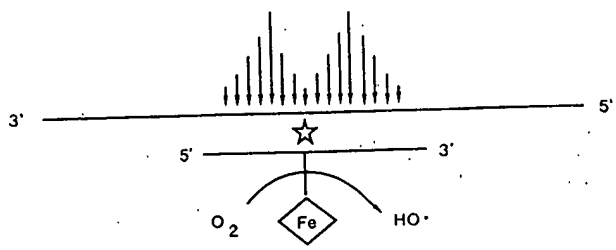


Figure 50. Intensities of cleavage by an artificial endonuclease (EDTA/Fe bonded via C^5 to thymidine) according to Dreyer and Dervan.[248]

2. Antisense Oligonucleotides for Specific Modification of the Target Nucleic Acids

Irreversible inactivation of the target nucleic acid may occur either in a catalytic process with cleavage of the internucleotide linkage (artificial endonucleases, ribozymes in sections V.A) or in a stoichiometric reaction with the target nucleic acid (chemical cross-linkers and photo-cross-linkers). Characterization of the products of the irreversible modifications allows the binding site of the oligonucleotide on the target nucleic acid to be checked.

(a) *Artificial Endonucleases.* This class of antisense oligonucleotides provides selective cutting of the target sequence and makes the mechanism based on endogenous RNase H activity redundant. Vlassov and coworkers were the first to report phosphate ethyl esters of homooligothymidylates with an EDTA unit (ethylenediaminetetraacetate) at the 5′ end.[244] Some further work with the EDTA group on phosphodiesters[233,245,248,249] and on methylphosphonates[59] have been published. In the presence of the reducing agent DTT (dithiothreitol) and Fe(II) and after the oligonucleotide has hybridized onto the target nucleic acid, cleavage of the latter is brought about by the EDTA unit. The chemical reaction is based on the local production of hydroxyl radicals. The extent of the reaction depends on the concentrations of oligonucleotide and salts and on the temperature.[233] After hybridization of a 16-mer oligonucleotide with a 5′-EDTA residue to a complementary 37-mer oligonucleotide, the cuts took place mainly within four nucleotide residues on either side.[245] Dreyer and Dervan incorporated the EDTA via C^5 of thymidine approximately in the middle of a 19-mer antisense oligonucleotide that had complementarity with pBR322.[248] After hybridization of this oligonucleotide to the appropriate heat-denatured fragment of pBR322, strand breakage was induced by addition of Fe(II), DTT, and O_2. Cleavage took place within 16 bases on either side (Figure 50).

Oligonucleotides modified with 1,10-phenanthroline[250,300,402] bring about, in the presence of Cu^{2+} and 3-mercaptopropionic acid, a hybridization-dependent cleavage of the target nucleic acid.

Schultz achieved selective RNA cleavage by linking staphylococcal nuclease, an extracellular enzyme composed of 149 amino acids, via the 3′ end to an oligonucleotide.[272,308] Surprisingly, hydrolysis took place

mainly at just one phosphodiester linkage. About 50% of the M1 RNA substrate that was used was cleaved after addition of the cofactor Ca^{2+}. It is evident that this strategy can function only in vitro, because it is not possible in vivo to eliminate Ca^{2+} using chelating reagents. Presumably, the oligonucleotide–nuclease conjugate would hydrolyze itself as a single strand in the presence of the cofactor.

A particular type of reaction is shown by the iron/porphryin system,[270,271] which oxidizes the heterocyclic bases, induces cross-linkages, and additionally causes strand breakages.

(*b*) *Chemical Cross-Linkers.* The cross-linking of DNA with oligonucleotides by attached alkylating agents have been investigated for some years by the Russian group of Knorre and Vlassov. Selective modification of nucleic acids at arbitrary positions had already been proposed by Grineva in their institute in 1967.[403] The derivatives that have been investigated most are those of (*N*-(2-chloroethyl)-*N*-methylamino)-benzylamide.[268,269,398,403–405] All four bases of the DNA are alkylated by 2-chloroethylamines, the reactivity decreasing in the sequence G > A ≈ C > T. The reaction takes place in two stages. Initially an ethylimmonium cation is formed by intramolecular cyclization, and it then attacks easily accessible nucleophiles. The position of the nucleotide which is attacked in the opposite strand depends on the type of attachment (3′ or 5′ end) of the reactive group on the oligonucleotide.[405] Oligonucleotides with alkylating radicals at the 5′ end react with the first nucleotide next to the complementary base, while those with alkylating groups at the 3′ end mainly attack the third nucleotide after the complementary base. However, there was also found to be reaction with a base that is 80 nucleotides further away in the sequence but is apparently in the spatial vicinity of the binding site.[405]

Webb and Matteucci have reported hybridization-dependent cross-linking using oligonucleotides that contain the reactive nucleoside 5-methyl-N^4,N^4-ethanocytosine.[205] This dispenses with the cyclization of the chloroethylamine derivatives, which is the rate-determining step with aromatic chloroethylamines. However, investigation of the ethano derivatives showed that the rate of alkylation with these compounds is much too slow to be useful.[205]

With regard to antiviral applications, mention should be made of the theoretical study of Summerton, which was submitted in September 1973 and finally published in revised form in 1978.[406] This article makes proposals on the treatment of viral infections based on chemically cross-linking oligonucleotides.

(*c*) *Photo-Cross-Linkers.* The cross-linking that can be achieved by irradiation, usually with long-wavelength light, represents an elegant method for controlling gene expression at a particular instant. Besides time-limited activation, another possibility is local activation, which might be achieved with fiber optic systems.[407] On the other hand, the distance tranversed by the radiation through organs is limited. Thus, photo-cross-linkers will be used mainly for eyes and skin and, with extracorporal irradiation, for the blood, too.

The most widely investigated derivatives are psoralen derivatives, which on irradiation with light of wavelength around 365 nm cause cross-linking to bases only of single-stranded DNA but not double-stranded DNA.[302] In the case of photoreaction with the DNA bases, alkali treatment results in strand breakage at the cross-link, which allows it to be located. Kean et al. found that the graded reactivity of methylphosphonate oligonucleotides with a 4′-(aminoalkyl)-4,5′,8-trimethylpsoralen residue at the 5′ end depends on the base in the complementary strand.[341] Thus, inhibition by psoralen paired with U is about 10 times better than that paired with C. Besides the intermolecular reaction, when there are two consecutive thymidine residues at the 5′ end, there must also be expected to be a intramolecular reaction.[341] Pieles and Englisch have reported that in the case of an 18-mer oligonucleotide with a trimethylpsoralen unit at the 5′ end the cross-linking obtained at 350 nm is photoreversible at 254 nm.[239] The mechanism of the photo-cross-linking is thought to be as follows: initially the bonded psoralen residues intercalate in the double strand. On irradiation with light at 360 nm two bases, preferentially thymine, react to give a cyclobutane adduct. Psoralen groups have been linked to phosphodiester[239,408,409] and methylphosphonate[59,302,341] oligonucleotides.

The group of *p*-azidophenacyl derivatives generate on irradiation (>300 nm) the very reactive nitrene radicals. They have been tested in combination with α-anomeric oligonucleotides.[193,243] In contrast to the psoralen derivatives, the azidoproflavines can also enter into reactions at double-stranded regions.[190,242] The limitations arising from this will be discussed in relation to the example of triplex formation (section V.B).

V. Modification of the Principles

Although the two principles described below are closely related to antisense technology, they form a separate class from the mechanistic viewpoint. Neither triplex formation nor ribozymes have been investigated with a view to therapeutic use as intensively as have the antisense oligonucleotides.

A. Ribozymes

Up until a few years ago it was generally believed that enzymes are always proteins. The works of Cech, Altman, Symons, Szostack, Uhlenbeck, and others, who have carried out intensive studies on catalytic nucleic acids in recent years,[410] have taught us better. Ribozymes are catalytic nucleic acids that often, as part of the mRNA structure, catalyze the self-splicing of the primary transcript. The ribozyme activity originally discovered by Cech in 1981[411] in *Tetrahymena thermophila* has not remained an isolated instance. Ribozyme activities in *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium*, plants, and eucaryotes suggest that they occur universally. Particularly of interest for therapeutic use are the hammerhead structures that occur in the viroids of plants. Forster and Symons attributed the self-cleavage activity of this satellite RNA to a hammerhead-like structure of the active site composed of only 55 nucleotides.[412] The hammerhead structure can be divided into two RNA fragments, a ribozyme and a substrate portion. Koizumi et al. synthesized two model oligonucleotides (21-mers) that contained the consensus sequences of the self-cleavage domains.[413] The substrate is cut in the presence of Mg^{2+}, resulting in a cleavage product with a 2′,3′-cyclophosphate resi-

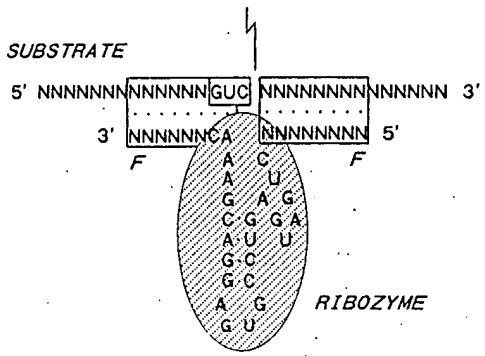


Figure 51. Consensus sequence of a ribozyme (F = flanking antisense region) according to Haseloff and Gerlach.[415]

due at the 3′ terminus. In another study, the same authors prepared ribozymes designed to cut any desired RNA sequences at predictable positions.[414] Thus, ribozymes might be formally included in the class of artificial endonucleases, with the consensus sequence GAAAC forming the RNase part and the two flanking sequences forming the antisense oligonucleotide part. Haseloff and Gerlach showed that ribozymes can be directed against any desired RNA sequences by flanking the conserved catalytic domains by appropriate antisense sequences (Figure 51).[415a] In most natural hammerhead RNAs the base triplet 5′ to the cleavage site is GUC. Alteration of GUC to GUA or GUU does not affect cleavage efficiency, whereas a change to CUC, AUC, or UUC results in a lower cleavage efficiency.[415a] Cleavage is strongly reduced for a GUG sequence 5′ to the cleavage site.[415b] Uhlenbeck prepared a 19-mer oligoribonucleotide by in vitro transcription with T7 RNA polymerase on a synthetic DNA template and used it for specific cleavage of a 24-mer RNA substrate prepared in a similar way.[416] The smallest ribozyme hitherto known is a 13-mer oligo*ribo*nucleotide.[417] By contrast, an analogous oligo*deoxyribo*nucleotide has no catalytic activity. However, it may be expected that certain ribonucleotide residues will be replaceable, at least in the antisense portion, by deoxyribonucleotide residues without loss of ribozyme activity.

Although the ribozyme strategy may appear very attractive at first sight, there are some fundamental problems. The rate constants for RNA catalysis are several orders of magnitude smaller than those of protein-catalyzed cleavage. In addition, the optimal cleavage temperatures are ≥37 °C.[417] It is likely that the dissociation and rehybridization of the ribozyme onto a new substrate molecule, which is necessary after cleavage of the substrate, take place too slowly for a catalytic action at low temperature. If the chosen flanking sequences are too long, dissociation becomes the determining factor, in which case only equimolar amounts of substrate are cleaved.[346] However, it is an advantage by comparison with artificial endonucleases that the ribozyme can become active only after hybridization.

B. Triplex DNA

In general, there are two possible ways to influence gene expression by binding an oligonucleotide to double-stranded DNA. On the one hand, the double-stranded DNA may partially melt and bind a complementary oligonucleotide with the formation of a duplex (cf. section IV.B). Another possibility comprises an

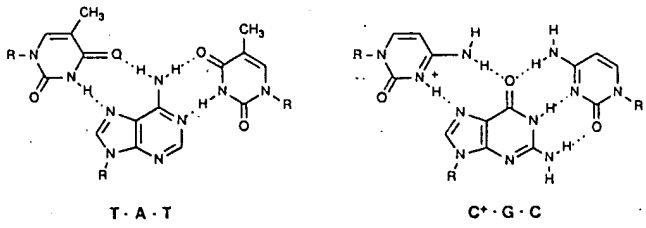


Figure 52. Triple base pairing according to Hoogsteen.

oligonucleotide binding to the double-stranded DNA with the formation of a DNA triplex structure. The antisense oligonucleotides that have been discussed previously belong to the first type, whereas the second type might be called "anti-gene oligonucleotide".[418] Felsenfeld et al. found, in 1957, the first triple helix in which two poly(C) strands hybridized with a poly(A) strand in the presence of $MgCl_2$.[419] Moreover, at pH 6.2 poly(U) forms with poly(G) a triplex structure in which one cytosine is evidently protonated. Figure 52 depicts both types of base triples. The Watson–Crick base pairing is supplemented by binding of a pyrimidine residue by Hoogsteen hydrogen bonds. Triplex structures are formed not only by homopolymers but also by repetitive sequences, for example poly(TC) with poly(GA). The ability to form triplex structures is greatly influenced by modification at the phosphate center. Thus, Miller et al. reported a hexathymidylate with an alternating methylphosphonate/phosphodiester backbone where one diastereomer forms a duplex with poly(A) whereas the other hybridizes in a triplex structure.[340] In the case of repetitive phosphorothioate oligonucleotides, the triplex formation depends on the position of the phosphorothioate residue.[298] A phosphorothioate 5′ upstream of purine promotes triplex formation, whereas one 5′ upstream of pyrimidine destabilizes a triple strand.

Double-stranded DNA can be modified or cleaved by triplex formation with derivatives having covalently bonded azidoproflavine,[190,243] EDTA/Fe,[249] chloroethylamine,[268] azidophenacyl,[243] or phenanthroline/Cu.[300] At present, however, application of this triplex formation appears to be confined to polypurine and polypyrimidine regions of DNA, which occur frequently in, for example, the control regions of eucaryotic genes. However, problems of specificity might arrive. There has merely been one report by Hogan that a 27-mer oligonucleotide with a mixed sequence inhibits the transcription of the *c-myc* gene.[372] A triplex structure was proposed, but this could not be proven experimentally.

Triplex formation is of great interest from two aspects. In the first place, inhibition at the level of transcription should be more efficient than at the level of translation, because many copies of mRNA are produced from 1 equiv of DNA. A pointer to a triple structure in Hogan's experiment is the high activity of the oligonucleotide in the 100 nM range, because unmodified oligonucleotides are usually active in the 10–100 μM range. In the second place, in the case of regulation at the DNA level, it is conceivable that there may be not only inhibition but also activation of transcription. It will certainly be of interest in the future to alter the heterocyclic base on the natural nucleosides in such a way that they preferentially form triplex structures irrespective of the particular sequence.

VI. Selection of Effective Target Sequences

A. Potential Target Sequences

The efficiency with which the function of a target sequence can be inhibited is closely connected to the mechanism of action discussed in section IV. Where the RNase H mechanism predominates, the inhibition efficiency will not depend on the binding site on the mRNA as long as this is easily accessible in the single-stranded form. As is evident from Figure 47, a large number of target sequences are suitable for inhibiting gene expression. At the level of translation, these are the 5′-non-coding region, the ribosome-binding site, the translation start region, the coding region, and the 3′-non-translated region. Potential points of attack at the level of transcription are the promoter, operator, attenuator, and terminator. Posttranscriptional targets are offered by the splice sites and the polyadenylation signals.

It is clear from most in vitro studies that antisense oligonucleotides act most efficiently when directed against the initial part of the 5′-non-coding region near the cap structure and against the region around the translation start codon.[252,290,317,420] These are better than the nontranslated region in between or the remaining coding region. The 3′-non-coding region is usually unsuitable for inhibition although it may in certain cases be a good target.[421] Thus, the best regions are those where the regulatory DNA-binding proteins are normally sited. Accordingly, it is particularly efficient to prevent binding of activators of gene expression and of translation-initiation factors. Similar conclusions regarding the target sequences have been derived from investigations on antisense RNA.[4]

When translation is inhibited by antisense oligonucleotides directed against the translation start region, there may in some circumstances be what is called the restart phenomenon. Normally, translation starts at an AUG codon. If an oligonucleotide directed against this region is used to suppress translation, it may start downstream at an internal AUG codon if this is preceded by a sequence resembling the ribosome-binding site. This may result in proteins shortened at the N terminus but still possibly functional. On inhibition of the G protein of VSV using methylphosphonate oligonucleotides directed against the start region, Engels noticed on a protein gel a concentration-dependent production of a new protein with a smaller molecular weight.[83] Similar restart problems also occur occasionally in expression of bacterial genes.[422-424]

In the case of HSV and HIV, oligonucleotides directed against particular splice sites on the RNA have proven particularly effective.[320,376] These results appear plausible on the basis of theoretical considerations, because splice sites must be readily accessible during mRNA processing. This is also true for the primer binding site of HIV, where reverse transcription of the viral genome starts.[376] Fine-tuning of the efficiency for various target sequences is not really possible because the variations in the relevant cell assay are too large.

B. Secondary Structure Considerations

Every mRNA has an individual secondary and tertiary structure that has a crucial influence on the efficiency of the target sequences. Although mR secondary structures can be calculated,[289,425,426] the ef ficiency of antisense oligonucleotides as inhibitors o protein translation has to be determined experimentall in practice.[394] Blake et al. have reported a considerabl effect of the secondary structure of the binding site o inhibition efficiency.[299] One possible way of detectin adverse effects from secondary structures is to degrad the mRNA with nucleases specific for single strands.[299,341] On inhibition of globin mRNA translation by psoralen-modified methylphosphonate oligonucleotides the cross-linking achieved with sequences directed against nuclease-sensitive regions was 10–30 times that obtained with those directed against nuclease-resistant regions.[341] Psoralen-derivatized oligonucleotides are thus also suitable tools for scanning mRNA secondary structures. However, indications of the presence of double-stranded mRNA regions can also be obtained from a simple cell-free in vitro translation test: if the efficiency of an oligonucleotide is increased by a previous hybridization reaction in which a solution of the oligonucleotide is heated with the mRNA to 90 °C and then cooled, this indicates secondary structures that can be eliminated by heat.

C. The Effect of Chain Length and Tandem Targeting

In the cell-free translation test, in which the passage of the oligonucleotide through the membrane plays no part, the efficiency of inhibition correlates directly with the oligonucleotide chain length.[290,303-305] This result suggests that the longest possible oligonucleotide should also be employed in the in vivo test. Two fundamental restrictions on this should be considered. First, large amounts of oligonucleotides can be readily synthesized only for the shorter ones (≤20 bases). Second, there is evidence that penetration through membranes diminishes as the chain length increases. These factors are the justification for employing two or more oligonucleotides that bind to adjacent regions of the mRNA, i.e., a tandem system. Maher and Dolnick investigated the inhibition of cell-free DHFR mRNA translation and found an unexpected synergistic effect by a factor of 3.5 on use of two tandem oligonucleotides.[305] Gaps of one or two nucleotides between the two oligonucleotides had scarcely any effect on this, whereas no synergism was detectable with gaps of more than 16 nucleotides. Goodchild et al. reported that a tandem comprising a 23-mer and a 25-mer oligonucleotide inhibited translation of globin synthesis in a cell-free system just as well as a corresponding 48-mer antisense oligonucleotide.[290] Only in one investigation has a mixture of two tandem oligonucleotides produced only the same inhibition as with each of the oligonucleotides separately.[303] The penetration characteristic gave rise to the hope that tandem oligonucleotides will show even more pronounced effects in vivo.

VII. Assay Systems

A. Cell-Fre in Vitro Translation

The advantages of cell-free test systems are the ease of manipulation and the fact that the results cannot depend on secondary effects such as penetration

of expression of the T-cell receptor has recently been demonstrated.[437] Kawasaki has reported inhibition of the expression of interleukine-2 in oocytes using 18- to 23-mer antisense oligonucleotides in the picomolar range.[431]

Research is most advanced in the antiviral area. Even the early studies by Zamecnik and Stephenson[14,15] looked at antisense oligonucleotides directed against Rous sarcoma viruses. In addition, it has been possible to inhibit the growth of vesicular stomatitis viruses,[255,321,347] herpes simplex viruses,[58,247,320] and influenza virus[262] in cell culture. Antisense oligonucleotide research has experienced a considerable expansion in recent years due to the world-wide AIDS problem. A meeting was recently organized by the National Cancer Institute (NCI) and National Institute of Allergy and Infectious Diseases in Maryland solely devoted to the topic of antisense oligonucleotides. Many of the publications on HIV inhibition originate from the Zamecnik group or from the NCI where unmodified oligonucleotides,[252,327,376] phosphorothioates and phosphoramidates,[102,319] and methylphosphonates[295] have been successfully tested against HIV (cf. section VIII). The target sequences that could be most efficiently inhibited proved to be the splice sites[327,438] and primer-binding sites[327] of HIV.

For the sake of completeness, mention should also be made of the applications of the antisense technique in agriculture. Although synthetic oligonucleotides can be used, for example, to inhibit the translation of the mRNA of potato virus X,[389] it will probably be more economic to express antisense RNA in plants.[439,440]

X. Future Prospects

Many studies have demonstrated that antisense oligonucleotides are taken up by live cells despite their polyanionic nature. Both unmodified oligonucleotides and their derivatives with a different charge and polarity are able to pass through the cell membrane and inhibit specifically the expression of genes inside the cell. Two different groups have demonstrated the in vivo efficacy of modified antisense oligonucleotides against viral infections in mice. No toxicity problems have yet emerged. However, more extensive in vivo studies will take place only after the synthesis of oligonucleotides has been optimized and made less costly. Simply to change from deoxyribonucleotide to ribonucleotide units might reduce the costs by a factor of 10–20. The protective group technique that has been largely taken over from gene synthesis is too elaborate and costly for synthesizing antisense oligonucleotides on a large scale. If template-controlled chemical polymerization of nucleotides proves to be a reasonable alternative, it might be possible to dispense entirely with protective groups. Another future possibility is amplification of synthetic DNA similar to the polymerase chain reaction.[441] However, the large sums that still have to be found for the preparation of large amounts of antisense oligonucleotides must not be a deterrent. This problem can be solved. The consequences of abandonning the development of a therapeutic agent in the very early phase because of price considerations can clearly be seen when looking at the development of penicillin where the original price was several orders of magnitude higher than now.

However, a therapeutic use of antisense oligonucleotides also demands a lowering of the effective concentrations, which in cell assays are now about 100 nM in favorable cases but tend on average to be 10 μM. An improvement in the activity by only 2 orders of magnitude ought to make therapeutic use of antisense oligonucleotides possible. Besides increasing the ability to cross membranes, it might be of major importance to achieve the correct localization of antisense oligonucleotides in the various compartments of the cell. If these problems can be solved, antisense oligonucleotides promise to open up a new era of drug research with the possibility of rational drug design based on the nucleotide sequences of the genes causing the disease.

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The Synthesis of Modified Oligonucleotides by the Phosphoramidite Approach and Their Applications

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Contents

INTRODUCTION

Although the application of nucleosidic and non nucleosidic phosphoramidite derivatives to the preparation and functionalization of oligonucleotides has recently been reviewed,[1,2] the synthesis of

oligonucleotide analogues *via* nucleosidic phosphoramidites and their applications will be addressed in this Report. Specifically, the relevance of modified oligonucleotides to the study of the physicochemical properties of DNA duplexes in solution and toward a better understanding of proteins-DNA interactions will be discussed along with experimental models pertaining to DNA damage and mutagenicity. Furthermore, the application of modified nucleoside phosphoramidites to the synthesis of oligonucleotide analogues as inhibitors of gene expression and as potential therapeutics will be emphasized. Given the efficacy of oligonucleotides as chemotherapeutics is largely dependent on their cellular permeation, their stability to extracellular and intracellular nuclease degradation, and their affinity to selected targets, the synthesis of oligonucleotides with modified internucleotidic phosphodiester linkages and/or carbohydrates will be reviewed in detail.

1. THERMODYNAMIC PROPERTIES OF DNA/RNA, PROTEIN-DNA INTERACTIONS, AND DNA/RNA RECOGNITION

1.1. DNA/RNA Thermodynamics and Protein-DNA Interactions.

The insertion of 7-deaza-2'-deoxyguanosine d(c^7G) *via* the phosphoramidite **1a** (Table 1) in the self-complementary hexamer d(c^7GCc^7GCc^7GC) generated a duplex having a lower T*m* than that obtained with an unmodified duplex (ΔT*m* = 10 °C).[3a] A possible explanation for this phenomenon could be the higher *pKa* of the N-H function of d(c^7G) (*pKa* = 10.3) relative to that of 2'-deoxyguanosine (*pKa* = 9.3) which resulted in a destabilization of the Watson-Crick d(c^7G)-dC base pair. Similarly, the phosphoramidites **2a-b** and **3a-b** were utilized in the preparation of modified alternating d(G-C)$_3$ sequences to probe the structure of these DNA duplexes.[3b]

Seela and coworkers demonstrated that the integration of 7-deaza-2'-deoxyadenosine [d(c^7A)][4a,b] or 8-aza-7-deazaadenosine [d(c^7z^8A)][4c] *via* **4a**, **5a-b** or **6** into oligonucleotides provided stabilization of the d(c^7A-T)$_3$, d(c^7A-T)$_6$, d(c^7z^8A-T)$_9$, and d(c^7z^8A-T)$_{12}$ duplexes relative to the corresponding d(A-T) sequences. The nature of the adjacent nucleobases was nonetheless important to the stabilization or destabilization of duplexes containing d(c^7A).[4a]

The incorporation of d(c^7G) at the cleavage site of the palindromic octamer corresponding to the recognition sequence of the endonuclease *Eco*RI [d(pGc^7GAATTCC)][5a] resulted in an enzymatic hydrolysis rate of less than 2% than that observed with the unmodified palindromic duplex.[5b] It was concluded that the *N*-7 of guanine was a binding site between the DNA fragment and the enzyme but was not a prerequisite for the recognition of the DNA segment. The substitution of d(c^7G) for dG lowered the T*m* of the duplex by 4 °C implying that the substitution had minor influence on its secondary and tertiary structure.[5b]

Numerous modified nucleosides represented by the phosphoramidites **3-30** (Table 1) have been incorporated into synthetic oligomers to evaluate duplex stabilities, and to provide a better understanding of the restriction site determinants required by *Bam*HI,[6a-b] *Dpn*I,[6d] *Eco*RI,[5,6a-c,7-10] *Eco*RV,[11-13] *Hin*dII,[14] *Hin*dIII,[15] *Sal*I,[14] *Sau*3A,[4c,7a] and *Taq*I[14] for sequence recognition and phosphodiester cleavage. In addition, the deoxyribonucleoside phosphoramidites **17** and **18** have been inserted in dodecamers to study ligand-DNA and protein-DNA interactions. The 5-methyl-4-pyrimidinone residues were resistant to the conditions used during the synthesis and deprotection of DNA sequences. However, the 2-thiothymine nucleobases decomposed to various products upon treatment with an iodine solution.[16b]

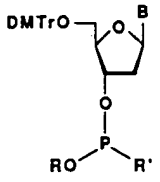
Diekmann and McLaughlin[17a] reported that the ligation of a decadeoxyribonucleotide containing the *Eco*RI recognition site formed a series of "curved" multimers on the basis of anomalous migration in polyacrylamide gels. It was shown that the insertion of modified base pairs in the *Eco*RI recognition sequence using the deoxyribonucleoside phosphoramidites **10b**, **19**, **28** or **29**, led to increased migration anomaly or increased DNA curvature when the purine substituent at *C*-6 was an amino group rather than a hydrogen or an oxo group. The deletion of the 2-amino group of guanine in the minor groove of the B-DNA helix was also effective at increasing the observed DNA curvature.[17a,b] It would appear from the endonuclease catalysis data, that curvature of the DNA helix axis is an inherent property of the

d(GAATTC) sequence which optimizes the interactions between *Eco*RI and its recognition sequence.[17a]

Hagerman has additionally demonstrated that the substitution of uracils for thymines in DNA containing short $(dA)_n(dT)_n$ sequences or the substitution of 5-methylcytosines for cytosines in $(dI)_n(dC)_n$ tracts (*via* the corresponding deoxyribonucleoside phosphoramidites) had a pronounced effect on the degree of stable curvature of the helix axis.[18] Such effects argued for a general structural perturbation due to the methyl group and it was thereby concluded that pyrimidine methyl groups could influence protein-DNA interactions not only through protein-methyl group contacts but also by locally altering DNA structure.[18] In spite of these findings, Diekmann *et al.* stated that the ability of a DNA sequence to adopt a B' structure with large propeller twist to confer intrinsic curvature on the helix axis was mainly determined by the stability of the stacking interactions between adjacent base pairs. Pyrimidine methyl groups can modulate the extent of curvature present but were not a dominant factor for the observed phenomenon.[19]

Phosphoramidites of 7-deaza-2'-deoxyadenosine $d(c^7A)$ (**4a**, **12a-b**) have also been employed to replace deoxyadenosine within $(dA)_6$ tracts to evaluate the effect of the isosteric nucleobase on the bending of oligonucleotides.[20] A decreased bending was observed upon substitution of $d(c^7A)$ for dA in these oligonucleotides. However, the decrease in bending was strongly dependent on the position of $d(c^7A)$ within the $(dA)_6$ tracts. These observations are in agreement with those reported by Ono *et al.* who described the incorporation of the 2'-deoxydeazaadenosine phosphoramidite **4b** or **11** at selected locations into $(dA)_4$-$(dT)_4$ tracts.[21]

Table 1. Deoxyribonucleoside Phosphoramidite Derivatives in the Study of DNA Thermodynamics and Protein-DNA Interactions



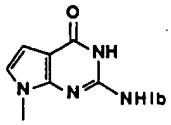
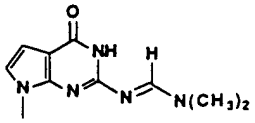
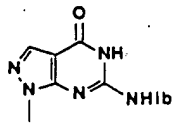
Compound	R	R'	B	References
1 a	CH_3—	—N(Pr-i)$_2$		3a-b,5a-b 8,35
b	$NCCH_2CH_2$—			
2 a	CH_3—	—N(Pr-i)$_2$		3b,68
b	$NCCH_2CH_2$—			
3 a	CH_3—	—N(Pr-i)$_2$		3b,7a,c
b	$NCCH_2CH_2$—			

Table 1. CONT'D

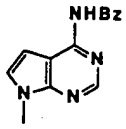
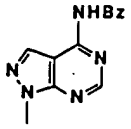
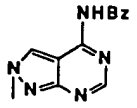
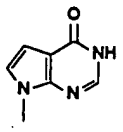
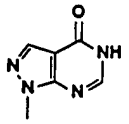
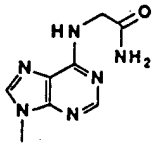
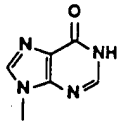
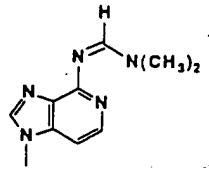
Compound	R	R'	B	References
4a	CH_3—	—$N(Pr\text{-}i)_2$		4a-b,5a,6a 7d,12a-b,20 21,34,69b
b	$NCCH_2CH_2$—			
5a	CH_3—	—$N(Pr\text{-}i)_2$		4c,6a,7a
b	$NCCH_2CH_2$—			
6	CH_3—	—$N(Pr\text{-}i)_2$		4c,7a
7a	CH_3—	—$N(Pr\text{-}i)_2$		5b,14 55a-b,68
b	$NCCH_2CH_2$—			
8	CH_3—	—$N(Pr\text{-}i)_2$		7a,55a-b
9	CH_3—	—$N(Pr\text{-}i)_2$		15
10a	CH_3—	—$N(Pr\text{-}i)_2$		13,14,17,19 34,35,55a-b
b*	$NCCH_2CH_2$—			
11	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		21

Table 1. CONT'D

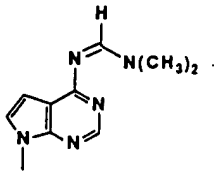
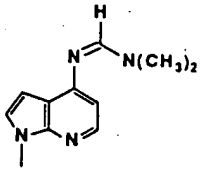
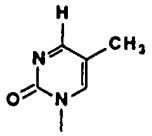
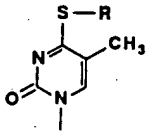
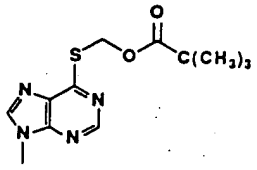
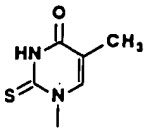
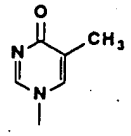
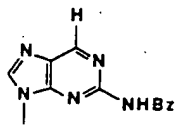
References	Compound	R	R'	B	References
4a-b,5a,6a 7d,12a-b,20 21,34,69b	12a	CH_3—	—$N(Pr\text{-}i)_2$		20
	b	$NCCH_2CH_2$—			
4c,6a,7a	13	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		27b
4c,7a	14*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a-b,16a 34,69a,70
5b,14 55a-b,68	15a R= SCH_3 b = Ph c = p-NO_2Ph d = CH_2CH_2CN e = CH_2OPiv	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a,b,d,69a 70-72
15	16	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		72
13,14,17,19 34,35,55a-b	17	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		12a-b,16b 70
21	18	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		16b
	19*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		6b,10 17b,69

Table 1. CONT'D

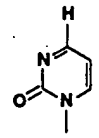
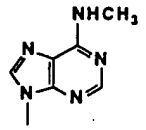
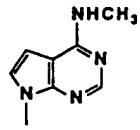
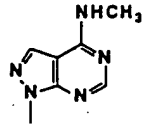
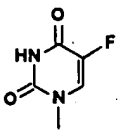
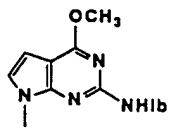
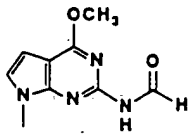
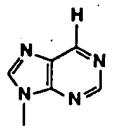
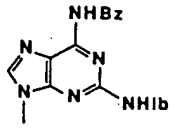
Compound	R	R'	B	References
20*	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		16a,69b
21a	CH_3—	—N⟨O⟩		6d,73
b	CH_3—	—$N(Pr\text{-}i)_2$		
c	$NCCH_2CH_2$—	—$N(Pr\text{-}i)_2$		35
22	CH_3—	—$N(Pr\text{-}i)_2$		6d
23	CH_3—	—$N(Pr\text{-}i)_2$		6d
24	CH_3—	—$N(Pr\text{-}i)_2$		6d
25	CH_3—	—$N(Pr\text{-}i)_2$		8
26a	CH_3—	—$N(Pr\text{-}i)_2$		7b
b	$NCCH_2CH_2$—			
27a	CH_3—	—$N(Pr\text{-}i)_2$		7b
b	$NCCH_2CH_2$—			

Table 1. CONT'D

References	Compound	R	R'	B	References
16a,69b	28*	$NCCH_2CH_2$—	—N(Pr-i)$_2$		9,12a,13,14 17,34,69a,74
6d,73	29*	$NCCH_2CH_2$—	—N(Pr-i)$_2$		17b
35 6d	30	$NCCH_2CH_2$—	—N(Pr-i)$_2$		12c

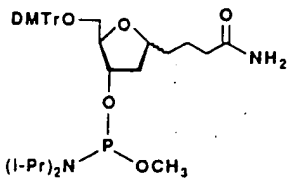
* The (9-phenyl)xanthen-9-yl group was also used as a 5'-*O*-protecting group.
ib= isobutyryl; Piv= pivaloyl; Bz= benzoyl

6d

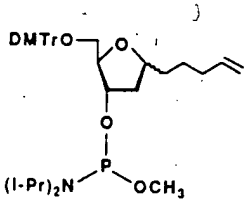
The 2-deoxy-D-ribofuranose phosphoramidite derivatives **31a-b** and **32** were prepared by François *et al.*[22] and applied to the synthesis of the oligodeoxyribonucleotide GTCGTGACYGGAAAAC, where Y represents the modified nucleotidic residue. Unexpectedly, a DNA duplex composed of the oligonucleotide analogue derived from the single insertion of **32** and its complementary sequence exhibited a higher T*m* than that of similar duplexes featuring either a pyrimidine-pyrimidine mismatch or a single incorporation of **31a** or **31b**.[22] It was speculated that the increased duplex stability resulted from an hydrophobic effect.

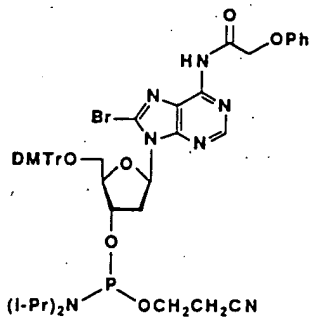
6d

8



7b





7b

31a α-anomer
b β-anomer

32

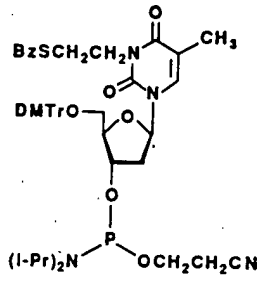
33

The 8-bromo-2'-deoxyadenosine phosphoramidite **33** has been synthesized and incorporated into oligonucleotides as a means to identify DNA nucleobase-amino acid contact pairs in protein-DNA complexes *via* photocross-linking techniques.[23a] Preliminary experiments showed that the presence of a

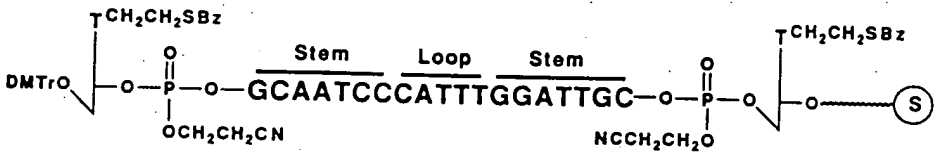
single photo-active 8-bromoadenine residue in oligoribonucleotides having a specific binding site for the transcription factor NF-κB has not significantly affected the stability of the corresponding DNA duplexes and their function as protein-binding sites.[23a] Consequently, 8-bromo-2'-deoxyadenosine-containing oligonucleotides may be useful in probing specific contacts in protein-DNA complexes.

A general method for the stabilization of the molecular architecture of DNA hairpins through disulfide bond formation has been proposed by Glick.[23b] The synthetic approach consisted of the consecutive preparation of N^3-benzoylmercaptoethyl-2'-deoxythymidine, the derivatization of a controlled-pore glass support with the modified nucleoside, and the incorporation of the modified phosphoramidite **34a** at the 5'-end of the oligonucleotide forming the hairpin structure (**34b**). Due to the unstability of the mercaptoalkylated thymine nucleobase to the harsh alkaline conditions employed for the removal of conventional base protecting groups, FOD-amidites[24] were used during solid-phase oligonucleotide synthesis. The purified mercaptoalkylated oligomer was aerobically stirred for 12 h to effect the predominant intramolecular formation of the disulfide link. The cross-linked hairpin DNA was isolated in 22% yield with a purity greater than 97%. The T*m* of the cross-linked hairpin was 21 °C higher than that of the unmodified hairpin.[23b] This synthetic methodology may be applicable to "trap" various conformations of the same sequence under controlled conditions.[23c]

It must be noted that the chemical synthesis of oligodeoxyribonucleotide dumbbells from deoxyribonucleoside phosphoramidites has also been undertaken to instigate detailed structural and physical studies of these macromolecules.[25]



34a

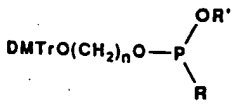


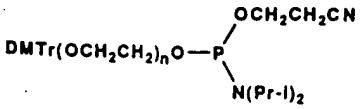
34b

A novel strategy for potential site-directed chemical reactions in DNA has been described by Asseline *et al.*[26] The approach involved the incorporation of a propanediol linkage (*via* **35a-b** or **36b**) at a defined location into an oligonucleotide and the subsequent generation of a DNA duplex with a complementary DNA strand having an extra nucleobase opposite the propanediol residue. Upon addition of an equimolar amount of a bis-acridine derivative, the stability of the duplex increased and NMR studies indicated that the bis-acridine intercalated the duplex at the site occupied by the propanediol residue.[26] These results suggest that this approach could target a specific chemical reaction, at a selected site in DNA, with a suitably derivatized bis-acridine derivative.

Seela and Kaiser[27a] employed the phosphoramidites **35a-b** to replace either dA or dT residues in the palindromic dodecamer d(CGCGAATTCGCG). The modified oligonucleotides exhibited a strong tendency to form hairpins presumably because of the highly flexible 1,3-propanediol linker.

The polyethylene glycol phosphoramidite **37b** has similarly been applied to the synthesis of the oligodeoxyribonucleotide d(GCTCACAAT-X-ATTGTGAGC), where X represents the hexaethylene





35a n = 3; R = N,N-diisopropylamino; R' = methyl
b = 3; = N,N-diisopropylamino; = 2-cyanoethyl
36a n = 2; R = morpholino; R' = 2-cyanoethyl
b = 3; = morpholino; = 2-cyanoethyl

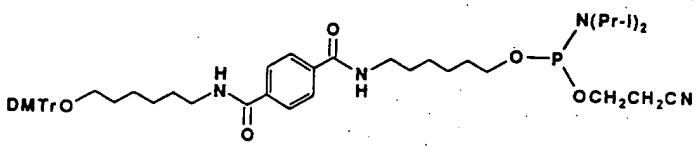
37a n = 2
b = 6
c = 10

glycol linker.[28a] Circular dichroism spectroscopy revealed that the oligonucleotide analogue existed as a bulged duplex and a hairpin structure in equilibrium with each other. Interestingly, the same oligonucleotide having X replaced with four thymines existed only as a hairpin structure. It was argued that the hairpin form was enthalpically stabilized by the stacking of the thymine residues of the loop region.[28a]

The incorporation of **37b** into the oligonucleotide $(dA)_{12}$-X-$(dT)_{12}$-X-$(dT)_{12}$ by solid-phase methods generated an oligonucleotide capable of folding back on itself twice to form a triple helix at low temperature.[28b] Thermal denaturation analysis revealed two cooperative transitions. At low-salt concentration (0.1 M sodium chloride), the transition of triplex to duplex with a dangling X-$(dT)_{12}$ extremity occurred at *ca.* 30 °C while the duplex to coil transition was observed at *ca.* 60 °C. CD spectroscopy indicated that the conformation of the triplex structure of $(dA)_{12}$-X-$(dT)_{12}$-X-$(dT)_{12}$ was essentially identical to that of a mixture composed of $(dA)_{12}$ + $2(dT)_{12}$.[28b] The facile formation of intramolecular triple-helical DNA structures relative to intermolecular triplexes provides a better opportunity to study the interaction of small molecules, such as intercalators, with these helices.

The terephthalamide phosphoramidite **38** has also been applied to the solid-phase synthesis of $(dT)_6$-X-$(dA)_6$ and $(dT)_6$-X-$(dT)_6$-X-$(dA)_6$, where X represents the terephthalamide linker.[29] The folding patterns displayed by these oligonucleotides were similar to those reported by Durand *et al.*[28a]

In this context, various ribonucleoside phosphoramidites have been used in the synthesis of oligoribonucleotides to investigate the structure and stabilizing factors of RNA hairpins[30] in addition to the thermodynamics of internal[31a] and bulge[31b] RNA loops.



38

Deoxy- and ribonucleoside phosphoramidites have been employed in the synthesis of chimeric DNA-RNA oligonucleotides.[32] These chimeras have shown subtle conformational effects resulting from the incorporation of a ribonucleotide into a DNA strand or the insertion of a deoxyribonucleotide in a RNA strand. To further study the conformational behavior of these modified oligonucleotides, chimeric duplexes have been synthesized and the structure of these duplexes has been analyzed by X-ray crystallographic techniques. These analyses revealed that [r(G)d(CGTATACGC)]$_2$, [d(GCGT)r(A)d(TACGC)]$_2$ and [r(GCG)d(TATACCC)/d(GGGTATACGC)] formed A-helices whereas [d(CG)r(CG)d(CG)]$_2$ formed a left-handed Z-helix.[32]

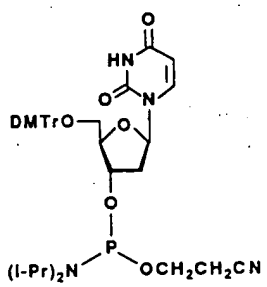
The insertion of modified nucleosides in oligonucleotides provided valuable insights toward a better understanding of the recognition of promoters by RNA polymerase in microorganisms. For example, Caruthers *et al.*[33] substituted uracil for either one or both adjacent thymine residues at position 34 and 35 relative to the transcription initiation site of the bacteriophage lambda P_R promoter. The loss of either methyl group reduced the rate of formation of transcriptionally competent complexes by 4-5 fold. The loss of the methyl group at both sites produced an inactive promoter. Consequently, the methyl group of these specific thymine residues appears critical for the interaction of RNA

polymerase with the lambda P_R promoter.[33b]

The interactions between the *trp* repressor and its operator sequence have similarly been studied by nucleobase analogue substitution.[34] The incorporation of the deoxyribonucleoside phosphoramidites **4b**, **14** and **28** at specific locations into oligonucleotides (20-mers) containing 18 base pairs of the *trp* operator was accomplished by standard solid-phase synthesis and afforded fourteen modified sequences. It was found that the carbonyl at dT_{+4} (the position of the center of symmetry of the doubled-stranded operator has been defined as 0) was critical for the formation of the high-affinity sequence-specific complex. In addition, the thymine methyl group at dT_{+4} and the *N*-7 of dA_{+5} appear necessary for high-affinity binding by the repressor. Interestingly, the deletion of the adenine amino group at dA_{-4} or dA_{+5} resulted in a sequence binding to the repressor with a higher affinity than that observed with an unmodified sequence.[34]

To better rationalize the high-affinity binding of HIV-1 *rev* protein to a bubble structure located within the *rev*-response element (RRE) RNA in stem-loop II, the incorporation of deoxyribonucleoside phosphoramidites (**1b**, **21c**, **28**, **39**) and ribonucleoside phosphoramidites (**40**, **41**) at selected positions into a minimal synthetic RNA duplex carrying the bubble was undertaken.[35] It has been speculated that high-affinity recognition of RRE RNA by *rev* requires hydrogen bonding to functional groups in the major groove of a distorted RNA structure.[35]

The insertion of 5-bromouridine (5-BrU) in oligoribonucleotides *via* the phosphoramidite derivative **40** has also been used to probe the interaction of MS2 coat protein with the translational operator of the MS2 replicase gene.[36] The increased stability of the complex formed between a specific 5-BrU operator and the coat protein relative to a wild-type operator presumably resulted from the formation of a transient covalent link between the modified operator and a cysteine side-chain of the protein *via* a Michael addition.[36]



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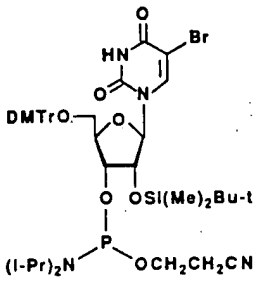

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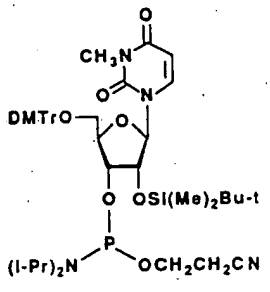
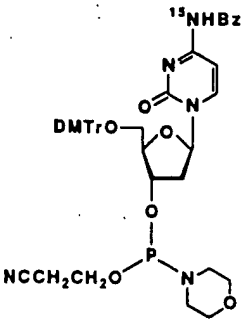


41

NMR studies of ^{15}N-labelled oligodeoxyribonucleotides have provided useful structural information about the nature of protein-DNA interactions.[37] ^{15}N-labelled phosphoramidites (**42** and **43**) were prepared and incorporated at specific locations into oligonucleotides corresponding to the symmetric 18 base pairs of the *lac* operator. The advantage of applying ^{15}N-edited NOE spectroscopy to map the individual environment of the protons coupled to the labelled nuclei was demonstrated and sequence-specific ^{15}N-chemical shifts were reported.[37a,b] Additionally, Massefski *et al.*[38] described the synthesis of the phosphoramidite **44** and its application to the solid-phase synthesis of a 17 base pair oligomer corresponding to the O_L operator of bacteriophage lambda. It was shown by "selective difference decoupling" that the three guanine residues of the oligonucleotide had different ^{15}N-chemical shifts.[38] Thus, the significant sequence dependence of ^{15}N-7 chemical shifts along with their sensitive detection through protons and facile assignments strongly support the utilization of $^{15}N7$-purine oligonucleotides in the study of DNA structure and dynamics.

A convenient synthesis of DNA segments having the exocyclic amino function of cytosine labelled with Nitrogen-15 has been reported by Kellenbach *et al.*[39] Their approach relied on the incorporation of the triazolo deoxyribonucleoside phosphoramidite **45a** into oligonucleotides and the subsequent



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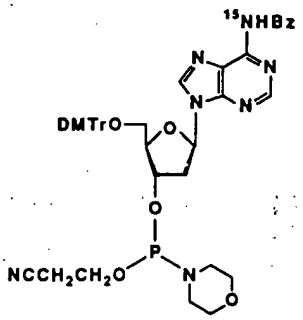

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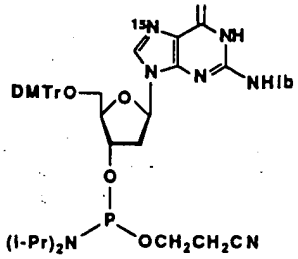
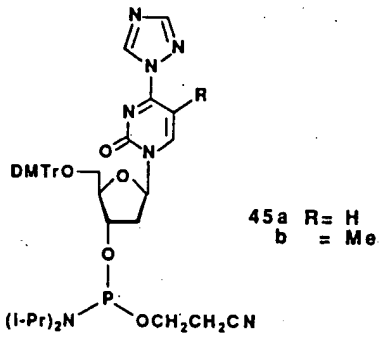


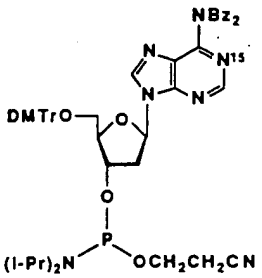
44

treatment of the modified oligomers with concentrated ^{15}N-ammonium hydroxide to generate $^{15}N^4$-cytosine residues. It must be noted that the ^{15}N-chemical shift of the exocyclic amino function of cytosine (*ca.* 98 ppm) is different than the ^{15}N-chemical shifts of proteins which usually resonate at 105-130 ppm.[39] The ^{15}N-labelling of the amino function of cytosines in oligonucleotides should consequently facilitate the study of protein-DNA interactions.



45a R= H
b = Me

^{15}N-NMR analysis of specifically labelled nucleic acids has been useful in probing local structural phenomena ranging from thermally induced local melting to the behavior of mismatched base pairs, and to the structural changes triggered by enzyme recognition or drug binding.[40] For example, the duplex formation of the self-complementary oligodeoxyribonucleotides d(CGT[$^{15}N^1$-A]CG) and d(CGT[$^{15}N^6$-A]CG) was reflected by an upfield shift of *ca.* 2.6 ppm for the N^1-resonance and a downfield shift of *ca.* 1.2 ppm for the N^6-resonance.[40] The *Tm* and thermodynamic data obtained for the helix-to-coil transition from the ^{15}N-NMR chemical shifts agreed well with those reported by other methods. The labelled phosphoramidite **46** or **47** was used in the solid-phase synthesis of the above self-complementary hexamers.[40]



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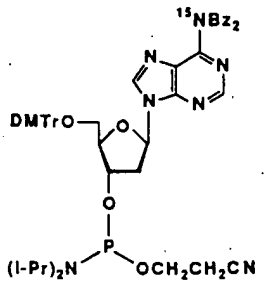

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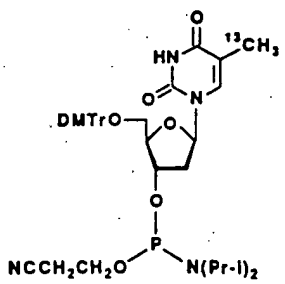
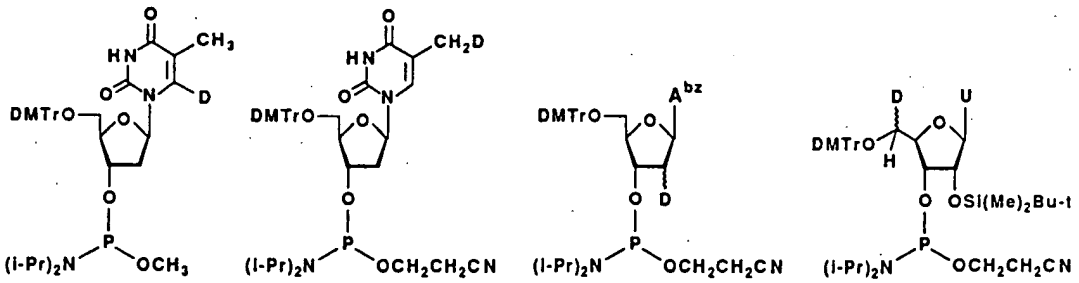


48

The deuterium-labelled deoxyribonucleoside phosphoramidites **49-51** have been incorporated at selected positions into the self-complementary dodecamer d(CGCGAATTCGCG). Such site-selective deuteration allowed the solid-state ^{2}H-NMR dynamic study of the nucleobases,[41a] methyl groups,[41b] and furanose rings[41c] in the DNA duplex.[41d] Similarly, the deuterium-labelled deoxyribonucleoside phosphoramidite **52** has been synthesized to enable the large scale preparation of a self complementary dodecaribonucleotide [r(CGCGAAU*U*CGCG) where U* is the deuterated residue] necessary for the study of RNA structure and dynamics.[41e]



49 **50** **51** bz= benzoyl **52**

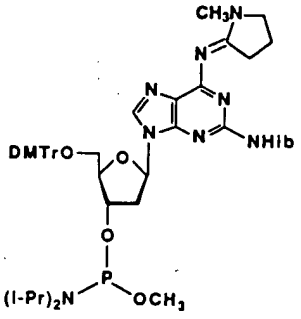
Carbon-13 relaxation is also a powerful probe for dynamic processes, as relaxation rates give accurate information about the motional characteristics of a particular C-H bond. A ^{13}*C*-6-labelled thymidine phosphoramidite has been synthesized by Williamson and Boxer[42a-b] and inserted in the oligonucleotide d(CGCGT*T*GT*T*CGCG) which adopted a hairpin conformation. NMR relaxation measurements indicated that subnanosecond internal motions were present in the loop region of the hairpin.[42b] Furthermore, the incorporation of the ^{13}C-labelled deoxyribonucleoside phosphoramidite **48** into an oligonucleotide corresponding to the stronger binding half-site of the consensus glucocorticoid response element has been reported.[43] The modified oligomer and its complementary sequence were interacted with the glucocorticoid receptor-DNA binding domain of the rat glucocorticoid receptor and the resulting complex was analyzed by NMR spectroscopy. The analysis revealed a hydrophobic contact between the labelled thymine methyl group and the methyl groups of a valine residue. These data demonstrated that stable isotope labelling of DNA functional groups in the major groove can simplify the NMR interpretation of protein-DNA complexes and provide more insight into the mechanisms underlying protein-DNA recognition.[43]

1.2. DNA and RNA Recognition.

Increased stabilization of DNA duplexes would permit more stringent hybridization conditions and should enhance probe specificity. In principle, the substitution of 2-aminoadenine for adenine would increase the stability of Watson-Crick base-pairing with thymine through the formation of additional hydrogen bonding between the 2-oxo group of the pyrimidine and the 2-amino function of the purine. Chollet *et al.*[44] reported the preparation of the protected 2,6-diaminopurine deoxyribonucleoside phosphoramidite **53** and its incorporation at selected positions into oligonucleotides by solid-phase synthesis. Tanaka *et al.*,[45] Brown *et al.*,[46] and Chazin *et al.*[47] also described the insertion of 2,6-diaminopurine in oligodeoxyribonucleotides by the phosphoramidite method. Hybridization probes containing 2-aminoadenine residues exhibited increased selectivity and strength in hybridization experiments with phage or genomic DNA. Each 2-aminoadenine contributed an additional 0.5-1 °C to the *Tm* of pentadecamer duplexes.[44a] In addition, ^{1}H-NMR analysis of a dodecanucleotide duplex having 2-aminoadenines instead of adenine nucleobases did not show disturbance of the global or local conformation of the modified DNA duplex relative to the native DNA duplex.[47]

Designing synthetic DNA probes from amino-acid sequences led Kong Too Lin and Brown[48a] to the development of the base-modified deoxyribonucleoside phosphoramidite **54** and its incorporation into oligonucleotides in an attempt to reduce probe multiplicity by removing the T/C degeneracy. The insertion of one or two such modified nucleobase(s) in two complementary 17-mers led to DNA duplexes as stable as the unmodified duplexes. However, the incorporation of three modified bases decreased the T*m* of the corresponding duplex by 6 °C. By comparison, an equivalent duplex with three similarly located mismatches exhibited a T*m* depression of 30 °C.

Oligonucleotides were also synthesized from the integration of the deoxyribonucleoside phosphoramidite **55**.[48b] The resulting DNA duplexes were not as stable (ΔTm = 6-13 °C) as those generated from the incorporation of **54**. It was speculated that the 5-methylene group or, perhaps, both methylene groups (in **54**) contributed to helix stability.[48a] Thus, deoxyribonucleosides derived from 6*H*,8*H*-3,4-dihydropyrimidino [4,5-*c*] [1,2] oxazin-7-one can base-pair with dG and dA and may find application in the preparation of hybridization probes and primers.[48c]



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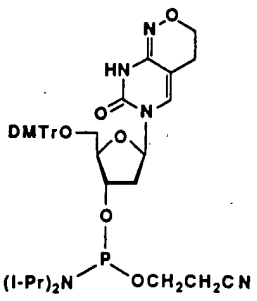

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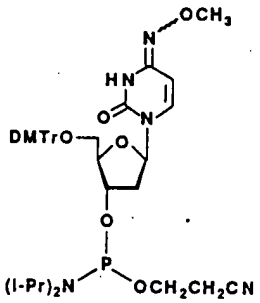
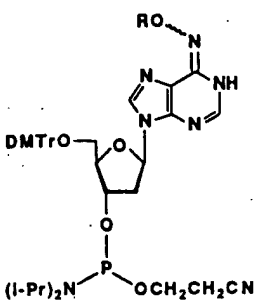


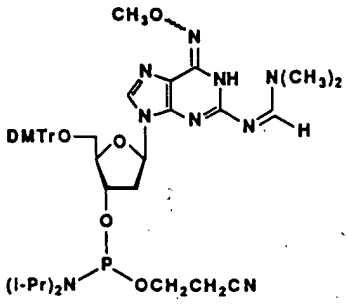
55

Subsequently, Brown and Kong Thoo Lin[49] described the synthesis of the deoxyribonucleoside phosphoramidites **56a** and **57** and their incorporation at selected sites into heptadecaoligodeoxyribonucleotides. It was shown that the purine nucleobase originating from **57** could form the most stable base pairs with a thymine or a cytosine of the opposite strand.[48c,49] Additionally, the triple insertion of **57** in oligonucleotides yielded duplexes of greater stability than those obtained with three purine-pyrimidine mismatches (ΔTm = 14 °C).[49] Furthermore, the single insertion of the phosphoramidite **56a** in a pentadecanucleotide indicated that the modified nucleobase (M) formed a relatively stable base pair with either thymine (T) or cytosine (C) of the complementary strand. The difference in stability between the (M:T)- and (M:C)-duplexes was small (ΔTm = 4 °C). These duplexes were however less stable than (A:T)- and (G:C)-duplexes, respectively, but were more stable than (A:C)- and (G:T)-duplexes.[50a-b] Under these conditions, the insertion of a N^6-hydroxyadenine residue (H) *via* **56b** generated a duplex having a (H:T) or a (H:G) base pair which exhibited a stability similar to that of the duplex carrying a (M:T) or a (M:G) base pair (ΔTm = 1-2 °C).[50c] Collectively, the incorporation of methoxyamino or hydroxyamino purines (from either **56a-b** or **57**) as "degenerate" nucleobases into DNA sequences may improve the efficacy of oligonucleotidic probes and primers.

It must be noted that the insertion of the 5-fluorodeoxyribonucleoside phosphoramidite **58** in oligonucleotides led to the formation of duplexes containing A:5-FU and G:5-FU base pairs that were considerably more stable than the corresponding duplexes having A:T and G:T base pairs.[51] Consequently, the synthesis of mixed hybridization probes for the detection of specific gene sequences can be alleviated by designing single hybridization probes containing 5-fluorouracil to pair with adenine or guanine, hypoxanthine[52] to pair with adenine or cytosine, and guanine to pair with cytosine or thymine at positions of codon degeneracy without significantly sacrificing duplex stability.[51] The chemical consequences of the incorporation of 5-fluorouracil into DNA (*via* the deoxyribonucleoside phosphoramidite **25**) have also been examined by NMR spectroscopy.[53]

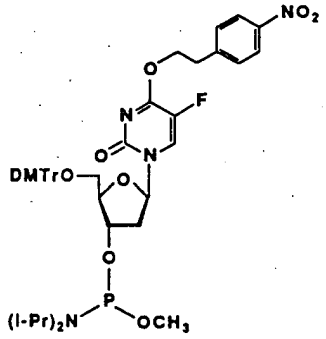


56a R= Me
b = Si(Ph)$_2$Bu-t



57

To evaluate the base-pairing abilities and mutagenicity of xanthine, the 2'-deoxyxanthosine phosphoramidites **59a-b** have been incorporated into oligodeoxyribonucleotides.[54] The thermal stability of each duplex containing xanthine base-paired with any of the four natural bases was determined and compared to that of similar duplexes carrying hypoxanthine. It was shown that xanthine-containing duplexes were as stable, at pH 5.5, as hypoxanthine-containing duplexes but were less stable than the latter at neutral pH.[54a] In addition, Seela and Kaiser[55a,b] reported the application of the isosteric 2'-deoxyinosine phosphoramidites **7a** and **8** in the synthesis of oligonucleotides to define the hybridization properties of these nucleobases. The single incorporation of the phosphoramidite **8** into the hexamer d(GCI*CGC) led to a DNA duplex having a T*m* lower (30 °C) than that of the unmodified duplex (T*m* = 46 °C) but higher than that of a similar duplex having hypoxanthine (T*m* = 27 °C). The phosphoramidite **8** may therefore become a useful synthon in the construction of hybridization probes containing an ambiguous base. Of interest, the purine nucleoside analogues corresponding to **7a** and **8** displayed an improved stability of the *N*-glycosidic bond relative to the unmodified purine nucleosides.[55b,c]



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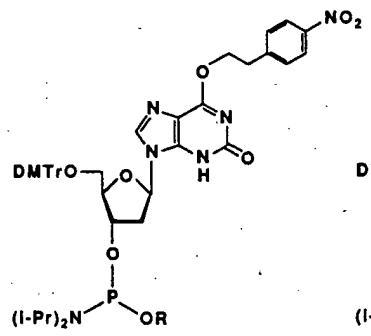
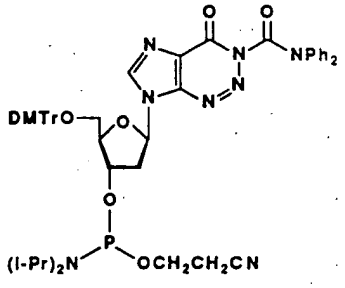


59a R= Me
b = CH_2CH_2CN

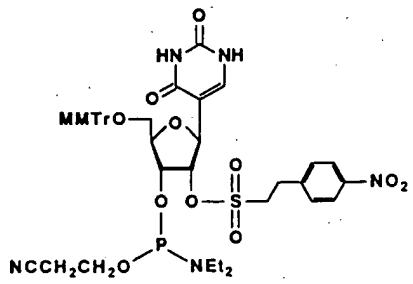


60

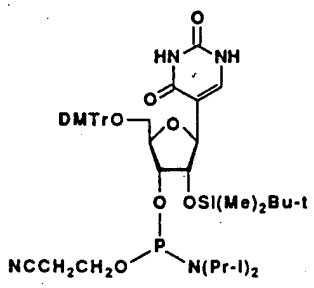
Fernandez-Forner *et al.*[56] disclosed the synthesis of the 2-aza-2'-deoxyinosine phosphoramidite **60** and its insertion in oligodeoxyribonucleotides to evaluate the stability of duplexes having this purine analogue base-paired with native nucleobases. Although the solid-phase synthesis of an oligonucleotide analogue proceeded well, the deprotection conditions (concentrated ammonium hydroxide, 40 °C, 2 days) promoted the decomposition of the 2-azahypoxanthine nucleobase and yielded an oligonucleotide containing a 5-amino-1-(ß-D-2'-deoxyribofuranosyl)imidazole-4-carboxamide (dAICA) residue.[56] Hybridization of the oligonucleotide analogue (19-mer) with a complementary DNA sequence carrying

a cytosine residue opposite the modified nucleobase produced a duplex having a depressed T*m* (48 °C) relative to that of an unmodified duplex under similar conditions (T*m* = 60 °C).[56] This approach represents, at least, an alternative to dAICA triphosphate and deoxyribonucleotidyltransferase for the incorporation of dAICA derivatives into oligonucleotides.

The pseudouridine phosphoramidites **61** and **62** have recently been applied to the automated synthesis of oligoribonucleotides.[57,58a,b] Specifically, two duplexes composed of AUAC Ψ Ψ ACCUG (Ψ represents pseudouridine), AUACUUACCUG and their complementary sequence CAGGUAAGUAU were prepared. These oligonucleotides corresponded to the 5'-end of human U1 snRNA paired to the mRNA consensus 5'-splice site. While the coupling efficiency of **61** or **62** was similar to that obtained with standard monomeric phosphoramidites, the free energy of duplex formation was found unchanged by the substitution of pseudouridine for uridine.[58a]

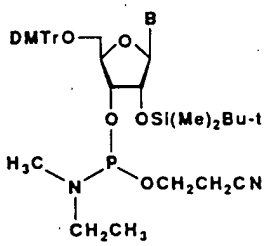


61



62

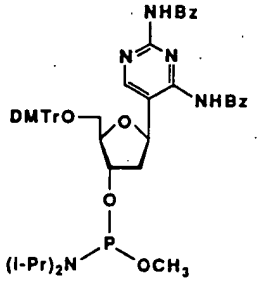
In a different context, the ribonucleoside phosphoramidites **63a-g** have been used in the total chemical synthesis of an *E. coli* $tRNA^{Ala}$ with its specific minor nucleosides (dihydrouridine, ribothymidine and pseudouridine). The phosphoramidites **63a-g** led to coupling yields greater than 98% within 2 min on a silica support.[58c,d] Triethylamine tris-hydrofluoride was found more effective than tetra-*n*-butylammonium fluoride for the complete removal of the 2'-*O*-silyl protecting groups. Following isolation and characterization, the full length tRNA exhibited a good aminoacyl acceptance activity.[58c,d]



63a B = uracil-1-yl
b = uracil-5-yl
c = thymin-1-yl
d = 5,6-dihydrouracil-1-yl
e = N^4-acetylcytosin-1-yl
f = N^6-phenoxyacetyladenosin-9-yl
g = N^2-phenoxyacetylguanin-9-yl

Novel nucleobases have been designed to expand the genetic alphabet from four to six letters.[59] Early experiments with *iso*-C and *iso*-G demonstrated that these modified nucleobases having novel hydrogen-bonding patterns were substrates for DNA and RNA polymerases.[59b] However, the chemical stability of these nucleobases affected the fidelity with which *iso*-C or *iso*-G was incorporated into oligonucleotides containing A and T residues.[59a] To circumvent this problem, the deoxyribonucleoside phosphoramidite **64** has been prepared and inserted in an oligonucleotide which served as a template for the Klenow fragment of DNA polymerase I. It was shown that 2'-deoxyxanthosine 5'-triphosphate was incorporated with high fidelity opposite the modified nucleobase and a full-length product was obtained.[59a] When applied to RNA, an expanded genetic alphabet should provide a greater diversity in the functional groups available to these molecules and, perhaps, increase their catalytic activities.[59a]

The synthesis of the 2'-*O*-methylpseudoisocytidine phosphoramidite **65a** and its integration into an oligothymidylate at two specific sites opposite the guanine residues in the duplex d(AAGAAGAAGAA)/d(TTCTTCTTCTT) has been reported.[60a] A mixture of this duplex with the modified oligothymidylate showed two thermal transitions between pH 7-8.7. One transition (Tm = 42 °C) corresponded to the denaturation of the duplex, whereas the the second transition (Tm = 12 °C) pertained to the dissociation of the modified oligothymidylate from the duplex. Interestingly, the second transition was not observed when deoxycytidine or 2'-*O*-methyldeoxycytidine was substituted for 2'-*O*-methylpseudoisocytidine in the oligothymidylate.[60a] Extension of this work to a 2'-*O*-methylpseudoisocytidine-containing hexadecanucleotide complementary to the "polypurine tract" found in the genome of human T-cell leukemia virus (HTLV-III) has recently been reported.[60b] It has been demonstrated that 2'-*O*-methylpseudoisocytidine can advantageously replace 2'-deoxycytidine in the formation of a triplex structure, as it can base-pair with 2'-deoxyguanosine in the Hoogsteen scheme without being protonated.



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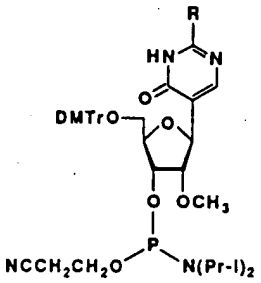
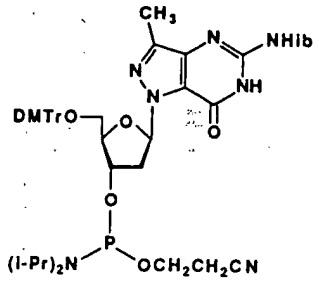


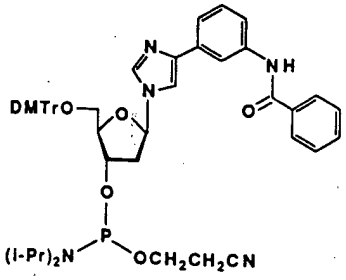
65a R= NHAc
b = N=CHNMe$_2$

In an attempt to design modified nucleobases that bind GC base pairs without protonation in a pyrimidine-motif triple-helical complex, Koh and Dervan[61a] reported the synthesis of the deoxyribonucleoside phosphoramidite **66** from ethyl(3-methyl-4-nitropyrazole-5-carboxylate) and 1-chloro-2-deoxy-2,5-di-*O*-*p*-toluyl-α-D-ribofuranose and its insertion in oligonucleotides at multiple interspaced or contiguous positions. It was shown by affinity cleavage analysis that an oligonucleotide carrying nucleobases derived from **66** was able to bind, at pH 7.8, a single fifteen base pair sequence of plasmid DNA containing five GC base pairs and, at pH 7.4, a single sixteen base pair sequence containing six contiguous GC base pairs. The nucleobase in **66** thus binds GC base pairs within a triple-helix motif with similar selectivity and strength as cytosine residues but over an extended pH range.[61a] This feature should increase the number of purine sequences amenable to oligonucleotide-directed triple-helix formation. Furthermore, the novel deoxyribonucleoside phosphoramidite **67** has been inserted in oligonucleotides to enable the selective binding of TA and CG Watson-Crick base pairs within a pyrimidine-purine-pyrimidine triple helix.[61b] When used in combination with the natural triplets TAT and C$^+$GC, this base-pairing specificity allowed oligonucleotide-directed sequence-specific recognition of double-helical DNA sequences containing all four base pairs at physiologically relevant pH and temperature without the need for an alternate strand crossover junction. It must however be noted that the sequence composition of target sites may influence the affinity of the modified nucleobase with TA and CG base pairs.[61b]

A strategy for the recognition of structured RNA has been proposed by Richardson and Schepartz.[62a] It was hypothesized that two oligodeoxyribonucleotides, linked by a tether, complementing two nonduplex and noncontiguous sites on *Leptomonas collosoma* SL RNA should cooperatively bind to the SL RNA with greater sequence and structure specificity than that of the two oligomers taken separately. The phosphoramidite **35b** was incorporated once or repeatedly (5 or 10 times) during solid-phase oligonucleotide synthesis to generate the tether between an heptamer (3'-



66



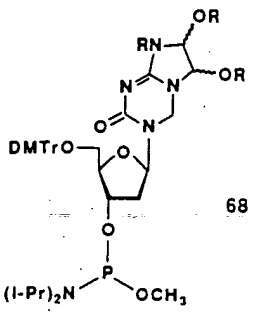
67

sequence) and a decamer (5'-sequence). The coupling efficiency of **35b** averaged 97%[62a] while, under similar conditions, the phosphoramidite **37c** exhibited a coupling efficiency of *ca.* 80%.[62b] Binding competition experiments with specific oligoribonucleotides and RNase H assays indicated that a tethered oligonucleotide probe interacted cooperatively and simultaneously with a single molecule of SL RNA. These probes are therefore attractive for the characterization and differentiation of tertiary structures in globular RNAs and ribonucleoproteins.[62a-b]

In a different context, two pyrimidine-rich oligodeoxyribonucleotides tethered with an hexaethylene glycol linker (derived from the phosphoramidite **37b**[28]) have been shown to form a stable triple-helix with a purine-rich single-stranded DNA sequence.[63] The formation of triple-stranded structures with single-stranded nucleic acids such as mRNAs, viral RNAs or DNAs may prove more efficient than double-helix formation at arresting translation, reverse transcription, or replication.[63]

The phosphoramidite **35b** has further served as a linker in the synthesis of homopyrimidine oligomers having opposite sugar-phosphate backbone polarities.[64] When appropriately linked, these oligonucleotide analogues can cooperatively form triple-helix structures with two or more homopurine segments located on the opposite strand of a target DNA duplex and stabilize the entire triple-helix relative to the corresponding unlinked homopyrimidine oligomers.[64]

Deoxyribonucleoside phosphoramidite derivatives have also been applied to the synthesis of circular oligonucleotides which have been found to form bimolecular triple-helical complexes with complementary DNA[65] and RNA[65b] oligonucleotides with high binding affinity relative to standard Watson-Crick complementary oligomers. Finally, several modified deoxyribonucleoside phosphoramidites have been incorporated into oligonucleotides to investigate either the base-pairing properties of an altered DNA template (as a mutagenesis model) with incoming deoxyribonucleoside triphosphates during DNA replication[54a] or the mechanism of mismatch repair in *E. coli*.[66] In another case, the deoxyribonucleoside phosphoramidite **68** led to the incorporation of 5-azacytosine residues at specific sites into oligonucleotides[67] in an effort to delineate the mechanism of DNA methyltransferase inhibition by triazines and its impact on the regulation of gene expression.

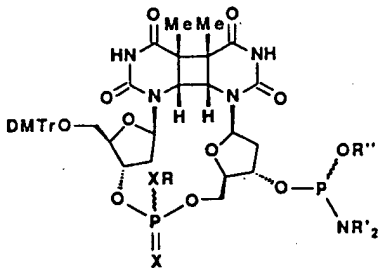


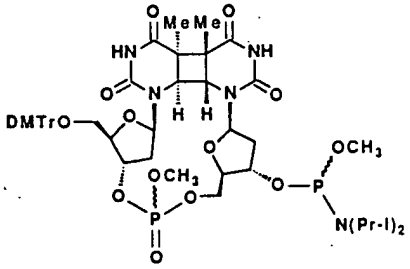
68 R= isobutyryl

2. DNA DAMAGE, DNA REPAIR, AND MUTAGENESIS

2.1. *DNA Photodamage*

It is well documented that exposure of DNA to ultraviolet light induces the formation of photoproducts at dipyrimidine sequences.[75a] Failure to repair such lesions has been associated with the genetic disease *Xeroderma pigmentosum* and skin cancer.[75b] Much of the work on mutagenesis by ultraviolet light and DNA repair has been hampered by the lack of well characterized DNA photolesions required for biological studies. To attenuate this limitation, Taylor *et al.*[76] and Murata *et al.*[77] described the synthesis of phosphoramidite building blocks composed of *cis-syn* (**69**,[76a] **70**,[77] and **71**[77]) and *trans-syn* thymine dimers (**72**[76b]). The sequence-specific incorporation of these dimeric phosphoramidites into oligonucleotides *via* solid-phase DNA synthesis was also described.





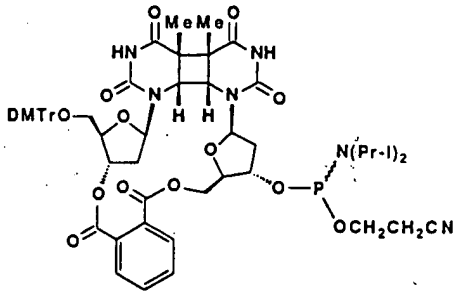
69 X= O; R= R''= Me; NR'_2= morpholino
70 = O; = CH_2CH_2CN; R'= i-Pr
71 = S; R= 2,4-dichlorobenzyl; = i-Pr
R''= CH_2CH_2CN

72

Two dodecamers derived from the insertion of **69** or **70** and **71**, respectively, were hybridized with their complementary sequences and each duplex was subjected to the endonuclease V of bacteriophage T4. It was shown that the phosphorodithioate link of the thymine dimer was cleaved by endonuclease V at a lower rate than that of the dimer having a natural phosphodiester linkage.[77] These data clearly indicated that the oligonucleotidic phosphoramidites **69**-**72** could be used in the preparation of unique photolesion-containing DNA duplexes for physical, enzymological, and mutagenesis studies.

In a different context, the phosphoramidite building block **73** has been prepared and centrally incorporated into an oligonucleotide (22-mer) by solid-phase synthesis.[78] Upon deprotection, the modified oligomer **74** was purified and submitted to photochemical and photoenzymatic cleavage experiments. Direct 254 nm photolysis of the modified 22-mer (**74**) led to the expected cleavage products (11-mers) in quantitative yield. Visible light photolysis in the presence of *E. coli* photolyase also generated the cleavage products to the same extent.[78] The ability to photochemically induce site-specific cleavage of nucleic acids may find application in the activation of prodrug forms of antisense oligonucleotides and ribosymes.

Fourrey *et al.*[79] reported the use of the triazolo deoxyribonucleoside phosphoramidite **45a** in the preparation of dimers containing thymine and 4-thiouracil residues. These dinucleotides were obtained by displacement of the 4-triazolyl function with hydrosulfide ion. The irradiation of the dimers with ultraviolet light resulted in the formation of (6-4)-pyrimidine-pyrimidone photoproducts. The mechanism of this DNA photodamage has been investigated[80] and the data supported the mechanism of (5-4)-bipyrimidine formation in tRNA containing 4-thiouridine. This information should prove useful in the study of the chemical behavior of related adducts in model systems and, ultimately, in double-stranded oligodeoxyribonucleotides.



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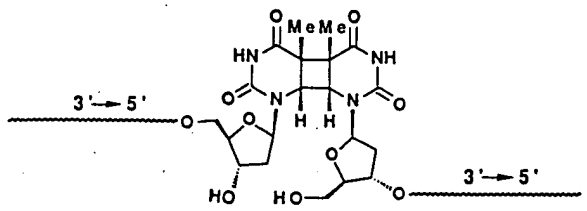


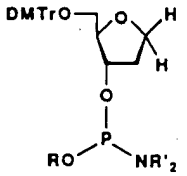
74

2.2. Apurinic/Apyrimidinic Lesions.

Biologically important lesions such as abasic (apurinic/apyrimidinic) sites in DNA arise spontaneously by a hydrolytic process which is accelerated by nucleobase modifications such as alkylation of purines and/or fragmentation of the heterocyclic rings.[75a,81a,b] The damaged bases are enzymatically excised by specific DNA glycosylases leading to the formation of abasic sites.[81a] It is well-known that these lesions represent a common structural intermediate in chemical mutagenesis, namely, a 2'-deoxyribose moiety linked through 3'- and 5'-phosphodiester bonds.[81b] In order to better understand the mechanisms involved in the repair of abasic sites in DNA, Takeshita *et al.*[82] inserted the tetrahydrofuranyl phosphoramidite **75** or the acyclic phosphoramidites **36a-b** at a defined position in an oligonucleotide to mimic the 2'-deoxyribose entity of abasic lesions. It was found that double-stranded oligodeoxyribonucleotides containing both types of abasic sites in one strand were endonucleolytically cleaved by *E. coli* endonuclease IV and exonuclease III. In contrast to exonuclease III, endonuclease IV did not cleave the DNA strand containing the abasic site generated from **36a**.

Synthetic oligonucleotides containing such abasic sites could also serve as templates for AMV reverse transcriptase, *E. coli* DNA polymerase I (Klenow fragment), *Drosophila* DNA polymerase-α, and calf thymus DNA polymerase-α.[82,83a] The extension of primer templates by these DNA polymerases led to the predominant incorporation of dAMP opposite any abasic site.[82,83a] These observations were consistent with those reported by Eritja *et al.*[83b] who employed the phosphoramidite **76a** for the incorporation of stable abasic sites into synthetic oligonucleotides.

In a different application, the insertion of the phosphoramidite **76b** in a synthetic c-Ha-*ras* gene demonstrated that abasic site analogues enhanced the transforming activity of the gene, relative to that of the unmodified oncogene, in transfected NIH3T3 cells.[84]

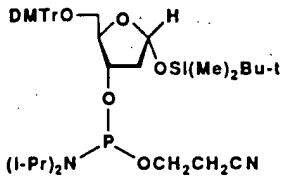


75 R= CH_2CH_2CN; NR'_2= morpholino
76a = Me; R'= i-Pr
b = CH_2CH_2CN; R'= i-Pr

The influence of the above abasic sites on the physicochemical properties of DNA duplexes[85] and triple-helical complexes[86] has been studied. Most of the structural aspects of these abasic site analogues appear relevant to the natural system except for the chemical reactivity of abasic DNA. In an attempt to synthesize oligodeoxyribonucleotides with genuine abasic sites, Groebke and Leumann[87] reported the preparation of the silylated 2-deoxy-D-ribofuranose phosphoramidite **77** in three steps from deoxy-D-ribose. The incorporation of **77** into oligonucleotides required a slightly longer reaction time than that of the natural deoxyribonucleoside phosphoramidites and occurred in yields greater than 95%. Although the abasic residue was stable throughout chain assembly, the removal of the nucleobase and phosphate protecting groups was performed with concentrated ammonium hydroxide in ethanol

(3:1) at ambient temperature. The purified oligonucleotide was then desilylated in a phosphate buffer (pH 2.0) within 1 h at 20 °C.[87] This easy protocol facilitated the synthesis of abasic DNA oligomers in large quantities regardless of the base sequence.

Subsequently, Vasseur *et al.*[88] and Péoc'h *et al.*[89] described the synthesis of the 2-deoxy-*D*-ribofuranose phosphoramidites **78a-b** and their application to solid-phase oligonucleotide synthesis. Typically, the ß-anomer **78b** led to coupling yields better than 98%. UV irradiation of the partially deprotected and purified oligomers in a 0.2 M ammonium formate buffer (pH 4) resulted in a rapid and complete cleavage of the *o*-nitrobenzyl protecting group. Oligomers up to 30 bases in length with natural abasic sites at selected positions have been synthesized according to this approach.[89] It must be noted, however, that the incorporation of the abasic phosphoramidite **78a** into oligonucleotides promoted the chemical cleavage of single-stranded DNA at specific sites under mild basic conditions.[90]



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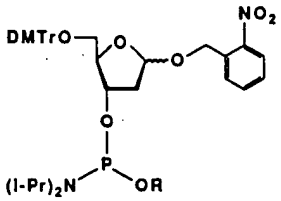


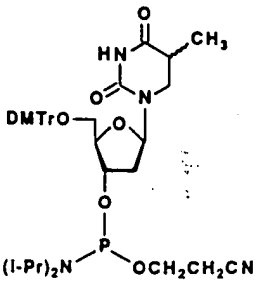
78a R= Me
b = CH_2CH_2CN

The 2-pyrimidinone phosphoramidites **14** and **20** (Table 1) have alternatively been used for the insertion of true abasic sites in oligonucleotides.[91] The coupling efficiency of **14** or **20** was similar to that obtained with the four common deoxyribonucleoside phosphoramidites.[16a] Incubation of a deprotected self-complementary dodecanucleotide containing a 2-pyrimidinone residue at pH 3 generated a DNA segment carrying an abasic site. The modified oligomer formed a DNA duplex exhibiting a much lower *Tm* (42.3 °C) than that obtained with the native self-complementary dodecanucleotide (*Tm* = 61.7 °C).[91] These data suggested that abasic sites may induce significant local destabilization in larger DNA macromolecules.

2.3. DNA Damage Caused by Ionizing Radiation.

It is well-known that gamma irradiation of HeLa cells with a cobalt-60 source in the absence of oxygen promotes the formation of 5,6-dihydrothymidine (DHT)[92] as the predominant 5*R*-diastereoisomer. However, when DNA is submitted to the action of gamma irradiation in aerated solution, the conversion of adenines to 7,8-dihydro-8-oxoadenines has been observed.[93] In order to investigate the mutagenic effects and the repair mechanism(s) of these DNA lesions, the synthesis of oligonucleotides carrying such defects at specific locations was necessary. The DHT phosphoramidite **79** was prepared in three steps from thymidine and was inserted in an oligonucleotide with a coupling yield of *ca.* 95%.[94a] Due to the sensitivity of 5,6-dihydrothymine to prolonged ammoniacal treatment, the phenoxyacetyl and isobutyryl groups were used for the protection of the amino function of purines and cytosines, respectively.[94b-d] The presence of DHT in the deprotected oligonucleotide was confirmed by partial chain cleavage occurring at the lesion site upon incubation of the oligomer with aqueous sodium hydroxide.[94a]

Similarly, the phosphoramidite **80a** and unmodified deoxyribonucleoside phosphoramidites carrying novel amino protecting groups[94b-d] have been applied to the synthesis of oligonucleotides ranging from 9-47 bases long.[95] The presence of modified adenines was verified by enzymatic digestion of the purified DNA segments.[95b,c] Oligodeoxyribonucleotides containing a single 7,8-dihydro-8-oxoadenine residue have been used as templates for *in vitro* transcription experiments with the Klenow fragment of *E. coli* DNA Polymerase I and the thermostable *Taq* DNA Polymerase from *Thermus aquaticus*. It was found that 7,8-dihydro-8-oxoadenine did not block replication, and thymine was incorporated opposite the damage.[95a]



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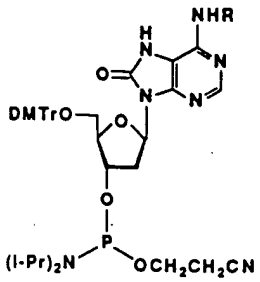
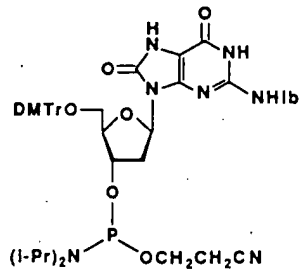


80a R= phenoxyacetyl
b = acetyl

The correlation between the formation of 7,8-dihydro-8-oxoguanine (8-oxoG) in DNA and carcinogenesis has been postulated.[96] To investigate the miscoding abilities of 7,8-dihydro-8-oxoguanine, the deoxyribonucleoside phosphoramidites **81**[97] and **82**[98] were prepared and introduced at defined positions in oligonucleotides. On primed templates, DNA polymerases directed the incorporation of either cytosine or adenine opposite the modified guanine residues.[99a] However, the frequency of mutations induced by 8-oxoG during replication *in vivo* was marginally above background in human cells. The most predominant mutation (1-2%) was a single G →T transversion. A higher frequency of this transversion (3-5 fold) was found in an excision repair deficient cell line. These data indicated that in contrast to the almost 100% mutagenicity generated by 8-oxoG with human polymerases *in vitro*,[99a] 8-oxoG was efficiently repaired *in vivo*.[99b] In addition, Wood *et al.*[99c] showed that the incorporation of 7,8-dihydro-8-oxoadenine into DNA *via* **80b** was at least an order of magnitude less mutagenic than that of 8-oxoG in *E. coli* cells with normal DNA repair capabilities.



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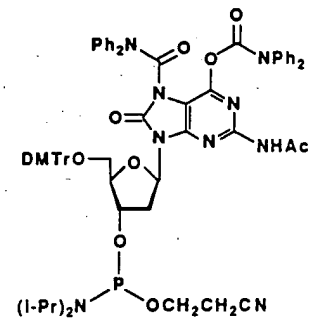
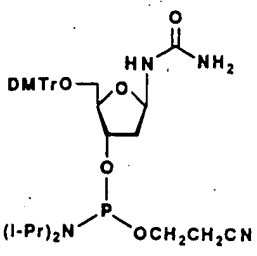


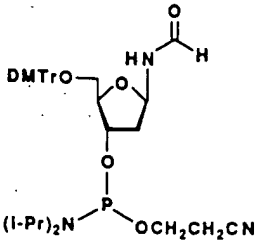
82

When DNA is submitted to ionizing radiation, deoxyribosylurea and deoxyribosylformamide were produced as fragmentation products of thymidine and 2'-deoxycytidine.[100a] The lack of oligonucleotides bearing one such lesion has made it difficult to study of the mutagenic activity of these defects and the repair mechanism(s) utilized by biological systems to correct these aberrations. Consequently, the synthesis of the deoxyribosylurea and deoxyribosylformamide phosphoramidites **83** and **84**, respectively, was performed and these monomers were integrated at specific locations in oligonucleotides ranging from 5 to 47 bases long.[100b-e] The instability of the ureido and formamido entities to alkaline conditions required phosphoramidites having phenoxyacetyl and isobutyryl protecting groups for the exocyclic amino function of the nucleobases during solid-phase synthesis of the modified oligonucleotides. The crude oligomers were purified by HPLC or preparative gel electrophoresis[100c] and the presence of the formylamino deoxyribosyl residue was confirmed by FAB mass spectrometry sequencing.[100d] Oligodeoxyribonucleotides having deoxyribosyl formylamine

moieties were used as templates for *in vitro* replication studies and it was demonstrated that the Klenow fragment of DNA Polymerase I directed the misincorporation of mainly guanine opposite the formylamino lesion.[100d]



83



84

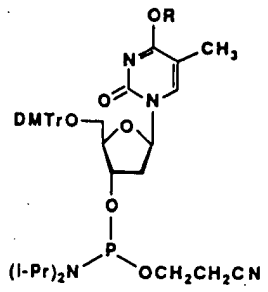
2.4. Alkylating Agents and Carcinogenesis.

The carcinogenicity of *N*-nitroso alkylating agents such as nitrosoureas and nitrosoamines is believed to be induced by O^6-alkylguanines and O^4-alkylthymines.[101a-b] Although, O^6-alkylguanines have attracted considerable attention in chemical carcinogenesis,[75a] the mutagenic potential of O^4-alkylthymines has been recognized[101c] and has been attributed to the very inefficient repair of O^4-alkylthymine residues in eukaryotic cells. To investigate the biological and structural role of O^4-alkylthymines in chemical carcinogenesis, the incorporation of these analogues into oligonucleotides has been undertaken. Because of the sensitivity of O^4-alkylthymines to acids, bases, and nucleophiles such as thiols or amines, the insertion of these modified nucleobases in synthetic DNA has been difficult. In spite of these drawbacks, Fernandez-Forner *et al.*[102a] developed a procedure for the solid-phase synthesis of oligonucleotides containing the mutagenic O^4-ethylthymine entity. Their approach entailed the preparation and utilization of the O^4-ethyl-2'-deoxyribonucleoside phosphoramidite **85a** in conjunction with deoxyribonucleoside phosphoramidites having nucleobase protecting groups similar to the *p*-nitrophenylethyl group.[102a,b] Due to the fragile O^4-ethylthymine residues, the modified oligomers were deprotected by treatment with 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine to remove nucleobase and phosphate protecting groups and then with 0.5 M DBU in ethanol/pyridine (1:1) to liberate the oligonucleotides from the solid support.[102a]

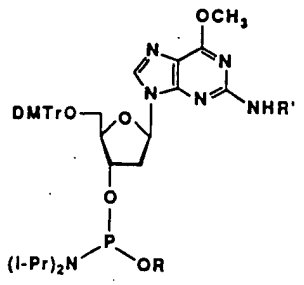
Along similar lines, Bhanot *et al.*,[103] Xu *et al.*,[104] Smith *et al.*,[105] Topal *et al.*,[106] Roelen *et al.*,[107a] and Pauly *et al.*[108a] reported the synthesis and application of deoxyribonucleoside phosphoramidite derivatives to the solid-phase synthesis of oligonucleotides containing O^4-alkylthymines[104] and/or O^6-alkylguanines.[103,104a,105,106,107a,c,108a] The O^4-alkylthymine phosphoramidite **85a** or **85b** was incorporated into oligonucleotides of up to 48 bases in length by routine solid-phase synthesis. The amino function of purines was protected with the phenoxyacetyl group while the amino group of cytosines was protected with the isobutyryl group to permit the complete deprotection of the oligomers with methanol/DBU or ethanol/DBU (9:1) and avoid the use of ammonia which is known to convert O^4-alkylthymines to 5-methylcytosines.[104b] Oligodeoxyribonucleotides containing O^6-methylguanines (from the incorporation of the phosphoramidite **86a** or **87a**) can, nonetheless, be deprotected by treatment with ammonia for two days at 20 °C without significant formation of 2,6-diaminopurines.[103,104a]

Roelen *et al.*[107b] and Xu *et al.*[107d] also reported the solid-phase synthesis of oligodeoxyribonucleotides containing O^4-alkylthymines. Their approaches consisted of the single incorporation of the triazolo-deoxyribonucleoside phosphoramidite **45b** at a defined position into oligonucleotides. Treatment of the fully protected and solid-phase bound oligomers with methanol, ethanol or *n*-propanol at 50 °C in the presence of DBU, yielded the corresponding oligonucleotides carrying O^4-methyl-, ethyl-, or *n*-propylthymine residues.[107b]

The 3'-*O*-(*N*,*N*-diisopropylamino)methoxyphosphinyl derivative of 5'-*O*-DMTr-O^4(O^2)-alkylthymidines or appropriately blocked O^6-alkyl-2'-deoxyguanosines has also been applied to the incorporation of *O*-alkylated nucleosides at selected positions into synthetic oligonucleotides.[108b-c] This



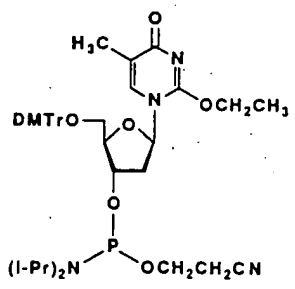
85a R= Et
b = Me



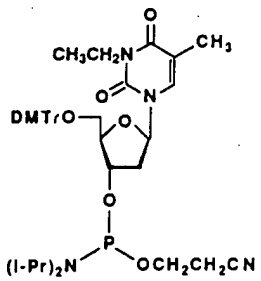
86a R= 2-cyanoethyl; R'= phenylacetyl
b = 2-cyanoethyl; = acetyl
c = 2-cyanoethyl; = isobutyryl
87a = methyl; = isobutyryl
b = methyl; = acetyl

type of monomeric phosphoramidites is not, however, recommended for the insertion of O^2-methylthymines in oligonucleotides, as the thiophenolate treatment required for the removal of the methyl phosphate protecting groups would also demethylate O^2-methylthymines. Interestingly, O^2-ethyl-, isopropyl-, and *n*-butylthymine derivatives were stable under similar conditions.[108b]

The O^2-ethylthymine deoxyribonucleoside phosphoramidite **88** has recently been prepared by Bhanot *et al.*[109] and inserted in an oligonucleotide by standard solid-phase synthesis with a coupling efficiency greater than 97%. The deprotection of the modified oligomer was performed using DBU/tetrahydrofuran/ethanol (14:43:43) for one week at ambient temperature. The purified oligomer served as a DNA template in the presence of T7 DNA polymerase and led to the incorporation of both dA and dT opposite the O^2-ethylthymine residue. While the incorporation of dA impeded DNA synthesis, the incorporation of dT resulted in efficient chain extension.[109] These data supported a molecular mechanism whereby an ethylating agent such as *N*-ethyl-*N*-nitrosourea can induce A→T transversion mutations contributing to cytotoxicity and, for example, to carcinogenic processes *via* the activation of proto-oncogenes. Similarly, the single insertion of the N^3-ethylthymine deoxyribonucleoside phosphoramidite **89** in a 17-mer produced a template for *in vitro* DNA replication experiments mediated by the Klenow fragment of *E. coli* DNA Polymerase I.[110] Like DNA lesions imparted by the incorporation of O^2-ethylthymines, DNA lesions incurred from the integration of N^3-ethylthymines contributed, in part, to the observed cytotoxicity and mutagenicity of ethylating agents.[110b]



88



89

The O^6-methylguanine deoxyribonucleoside phosphoramidites **86b** and **87b** have also been inserted in oligonucleotides by solid-phase synthesis.[111a,b] Typically, the coupling efficiency of **87b** averaged 91%.[111a] The random incorporation of a single O^6-methylguanine residue into self-complementary dodecanucleotides led to the formation of duplexes having *Tm* values lower than that observed with the corresponding unmodified oligomers (ΔTm = 19-26 °C). These data indicated that

the O^6-methylation of guanine generated regions of localized instability in DNA regardless of the base opposite the lesion. Such instability may disrupt critical regulatory events and may be as responsible as the mutation itself for triggering oncogenic processes.[111a] Furthermore, the insertion of O^6-methylguanine phosphoramidites in DNA sequences corresponding to either the region of the *c-fos* promoter containing the binding site for serum response factor (SRF), the region of the HIV LTR containing two binding sites for the transcription factor NF-κB, or the region of the HIV LTR containing three binding sites for the cellular factor SP1, inhibited the binding of cellular factors to these DNA sequences. It was additionally discovered that the binding inhibition resulted from only certain modified guanines within a given binding site.[112] These findings suggest that the alkylation of O^6-guanines may seriously affect the regulation of gene expression at the transcription level. It must also be noted that, consistent with these results, the incorporation of O^6-methylguanine (*via* **86c**) into codon 12 of a synthetic c-Ha-*ras* gene led to significant induction of focus formation upon transfection of the modified *ras* gene into normal NIH 3T3 cells.[113]

2.5. *Polycyclic Aromatic Hydrocarbon-DNA Adducts.*

The occurrence of carcinogenic or mutagenic effects associated with polycyclic aromatic hydrocarbons has been correlated with the ability of electrophilic metabolites to modify chromosomal DNA and interfere with the normal biochemical events involved in cell replication.[114a] Several lines of evidence suggest that these electrophiles preferentially react with the exocyclic amino group of guanine.[114b] Whether this preferential reaction occurs from optimal positioning of the aromatic electrophile *via* initial binding within one of the DNA grooves or by transient intercalation of the aromatic ring within the DNA double helix, is still unclear.[115] In order to provide a better understanding of the interactions of electrophilic polycyclic aromatic hydrocarbons with nucleic acids, the synthesis of oligonucleotides carrying well-characterized adducts, at selected sites, has been reported by Casale and McLaughlin.[115] The anthracenyl deoxyguanosine phosphoramidite derivative **90** was prepared from 2'-deoxy-4-desmethylwyosine and incorporated into oligonucleotides by solid-phase synthesis. The coupling efficiency of **90** was modest (65-70%) in spite of an extended condensation time (*ca.* 60 min). The circular dichroism spectrum of a DNA duplex (12-mer) having two anthracen-9-ylmethyl residues (one in each strand) failed to reveal a well-defined helical structure. Conversely, a duplex (13-mer) having only a single polycyclic aromatic residue existed essentially as a normal B-form DNA but exhibited a lower T*m* (50.6 °C) than that of an unmodified duplex (T*m* = 56.6 °C). The lack of substantial fluorescence quenching and the observed destabilization of the double-stranded 13-mer indicated that the covalent alkylation of DNA by electrophilic aromatic hydrocarbons may not result from the intercalation of the polycyclic aromatic electrophile. Exterior groove binding may be responsible for the initial association and/or positioning of the aromatic hydrocarbon prior to the alkylation of duplex DNA.[115]

The 3'-*O*-phosphoramidite derivative of N^6-(7-methylen-12-methylbenz-[*a*]-anthracenyl)-5'-*O*-DMTr-2'-deoxyadenosine has also been inserted in oligonucleotides in an attempt to determine the effect of the bulky polycyclic aromatic hydrocarbon group on the conformation and duplex stability of these oligomers.[116] A duplex composed of a pentadecamer carrying one aralkylated residue and a complementary unmodified sequence had a lower T*m* (46 °C) than that obtained with a native DNA duplex (T*m* = 55 °C) under the same conditions. The CD spectra of unmodified and aralkylated duplexes showed that the conformation of these duplexes was not significantly different from each other in solution.[116]

To further probe the mechanism(s) of cell transformation by polycyclic aromatic hydrocarbons, epoxide and diol epoxide adducts of these species at the exocyclic amino function of deoxyadenosine and deoxyguanosine have recently been synthesized and incorporated into oligonucleotides through the phosphoramidites **91a-d** and **92**.[117] The nucleosidic precursors to these phosphoramidite derivatives were 6-fluoro-9-(2-deoxy-β-D-*erythro*-pentofuranosyl)purine[117a,b] and 2-fluoro-2'-deoxyinosine.[117c] It has been shown that the interaction of the nonanucleotide d(GGTCA*CGAC), where A* represents the altered base (derived from **91d**), with the complementary strand d(CTCGTGACC) generated a duplex having a T*m* of 23 °C at low ionic strength. Under similar conditions, an unmodified DNA

duplex exhibited a
unchanged when d
(T*m* = 14 °C) was
therefore be intere
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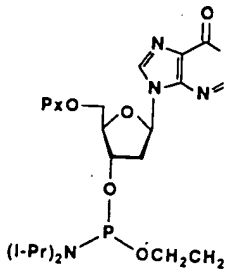


90

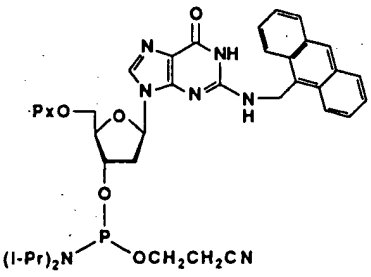
Harris *et al.*[1
phosphoramidites **93**
oligonucleotides cont
treatment of solid-ph
dihydroxy-*anti*-9,10-ep
8,9,10,11-tetrahydro-[*a*
corresponding adducts

DMT

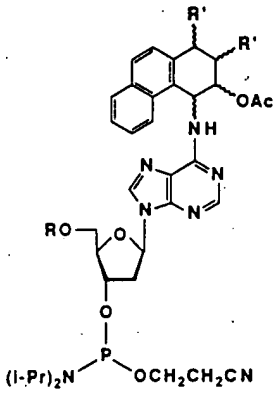
(I-Pr)

Of additional int
the 2'-*O*-(1-pyrenylmeth
defined locations into
pyrenylmethyl-oligonuc

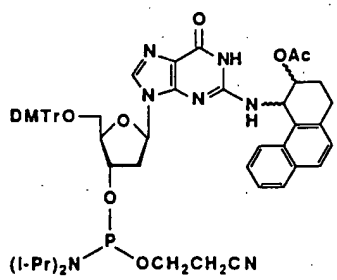
duplex exhibited a T*m* of 43 °C.[117d] Surprisingly, the thermal stability of the modified duplex was unchanged when dG, instead of dT, was opposite the adducted-adenine residue. A less stable duplex (T*m* = 14 °C) was nevertheless obtained when dA was opposite the modified adenine.[117d] It would therefore be interesting to determine whether such preference for specific mismatches in these DNA duplexes is a good predictor of nucleotide misincorporation during replication of the modified DNA.



90

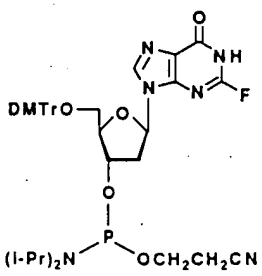


91a (3*R*, 4*R*); R= Px; R'=H
b (3*S*, 4*S*); R= Px; R'= H
c (3*S*, 4*R*); R= DMTr; R'= H
d (1*R*, 2*S*, 3*R*, 4*S*); R= DMTr; R'= OAc

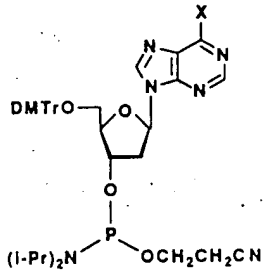


92 (3*S*, 4*S*)

Harris *et al.*[118] have reported the insertion of the halogenated deoxyribonucleoside phosphoramidites **93** and **94a-b** in oligonucleotides as an alternate approach to the preparation of oligonucleotides containing polycyclic aromatic hydrocarbon adducts. Their strategy consisted of the treatment of solid-phase bound halogenated oligonucleotides with amines derived from (±)-*trans*-7,8-dihydroxy-*anti*-9,10-epoxy-8,9,10,11-tetrahydro-[*a*]-pyrene or (±)-*trans*-8,9-dihydroxy-*anti*-10,11-epoxy-8,9,10,11-tetrahydro-[*a*]-anthracene which afforded, under the recommended conditions, the corresponding adducted oligomers.[118]



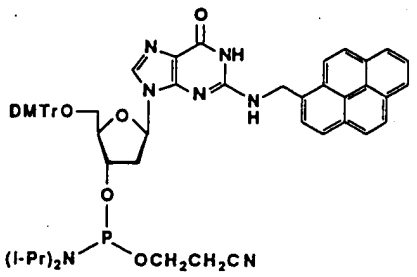
93



94a X= Cl
b = F

Of additional interest, *N*-pyrenylmethyl deoxyribonucleoside phosphoramidites (**95**, **96**)[119a] and the 2'-*O*-(1-pyrenylmethyl)uridine phosphorodiamidite **97**[119b-d] have been prepared and incorporated at defined locations into oligonucleotides by conventional solid-phase synthesis. Particularly, the pyrenylmethyl-oligonucleotide U*TTTTTTTTT, derived from the single insertion of **97** (designated by

U*) at the 5'-terminus of an oligothymidylate, hybridized to poly rA and produced a complex having a slightly higher T*m* (25.1 °C) than that of the corresponding oligothymidylate-poly rA hybrid (T*m* = 23.5 °C) under the same conditions.[119b,c] Unexpectedly, the fluorescence intensity of the pyrenylmethyl-oligonucleotide increased upon complexation with poly rA. In fact, the fluorescence of the complexe U*TTTTTTTTT-poly rA was *ca.* 49 times larger than that of U*TTTTTTTTT. This feature should lead to increased sensitivity in the detection of sequence-specific gene probes.[119d]



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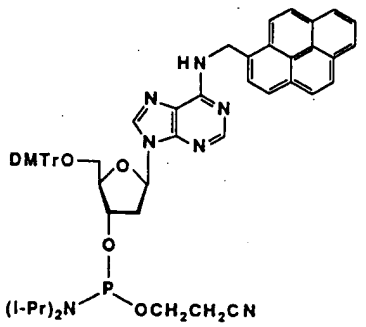
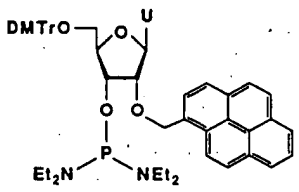
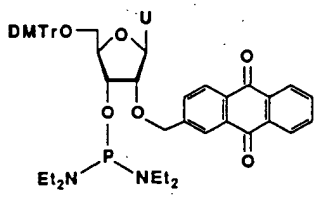


96

The synthesis of the anthraquinonylmethyl ribonucleoside phosphoramidite **98** from N^3-benzoyluridine has been reported by Yamana *et al.*[120] The incorporation of **98** into a self-complementary oligonucleotide (CCU*AGCTAGG) occurred with a coupling efficiency of *ca.* 87% according to a manual solid-phase synthesis protocol. The purified oligonucleotide formed a duplex exhibiting a T*m* of 57.4 °C in a buffer containing 0.1 M sodium chloride and 0.01 M sodium phosphate (pH 7.0). Under identical conditions, the unmodified DNA duplex had a T*m* of 40 °C.[120] It has been postulated that the increased stability of the modified DNA duplex resulted from the intercalation of the anthraquinone moiety between adjacent base pairs.



97



98

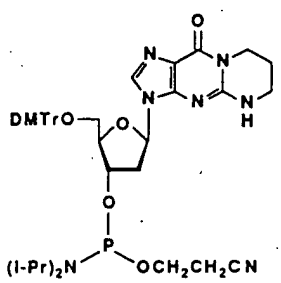
2.6. Acrolein-DNA Adducts.

Recent work has established that the mutagenicity of acrolein, a substance ubiquitous in the human environment, and other α, ß-unsaturated bifunctional carbonyl compounds could be due either to the formation of cross-links in DNA or to the formation of exocyclic adducts with one or more of the nucleic acid bases.[121] To better understand the effects created by acrolein-2'-deoxyguanosine adducts on replication *in vitro* and *in vivo*, the synthesis of oligonucleotides containing a specific exocyclic adduct was conducted *via* the incorporation of the 1,N^2-(1,3-propano)-2'-deoxyguanosine phosphoramidite **99** by standard solid-phase synthesis.[122] It is believed that the modified nucleobase of these oligonucleotides has preserved the major steric elements of both major and minor DNA-acrolein adducts and could, therefore, mimick these adducts in DNA replication studies.[122b]

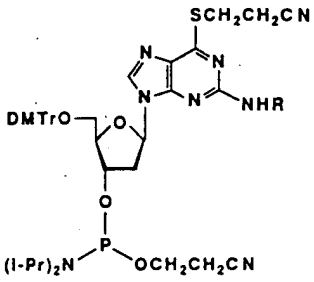
2.7. The Cytotoxicity of 6-Thioguanine and 5-Hydroxymethyl-2'-Deoxyuridine.

The cytotoxicity of 6-thioguanine has been exploited for years in the treatment of leukemias and

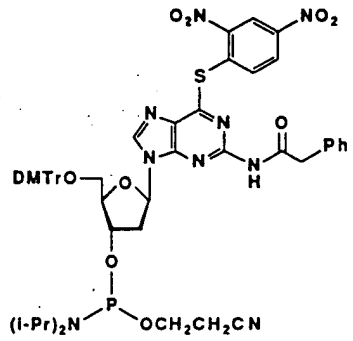
other human malignancies[123] but the molecular basis of its action is not clearly understood. It has, nonetheless, been argued that the incorporation of 6-thioguanine into DNA was responsible for its cytotoxicity. In an attempt to properly study this problem, Christopherson and Broom[124a] and others[69,124b,125a] devised the synthesis of 6-thioguanine-2'-deoxyguanosine phosphoramidites (**100a**,[124a] **100b**,[69,124b] **101**[125a]) and their incorporation into self-complementary oligonucleotides. It was shown that a dodecameric DNA duplex containing one or two 6-thioguanines (one in each strand) exhibited a significantly lower T*m* than that of the unmodified duplex (ΔTm = 6.9 °C or 12.8 °C, respectively).[125a,b] These findings prompted speculations about distorsions being created by 6-thioguanines in the DNA structure. Such distorsions could have been responsible for the observed decrease in stability of DNA duplexes and could, conceivably, account for the cytotoxicity of these drugs.[125a]



99



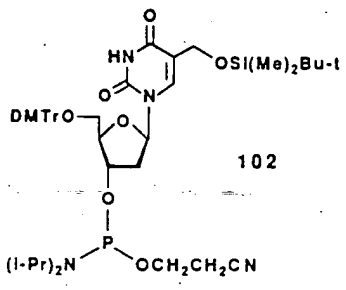
100a R= phenoxyacetyl
b = benzoyl
c = isobutyryl



101

In a different context, the incorporation of 6-thioguanines into short guanine-rich oligonucleotides may inhibit self-association and the formation of stable quadruplexes (G-tetrads) by interfering with hydrogen bonding and the coordination of alkali metal ions.[126] Given these properties, 6-thioguanine-containing oligonucleotides (*via* **100c**) may become more efficient in the formation of triple helices toward the inhibition of DNA replication. Interestingly, oligonucleotides containing 6-thioguanine residues exhibited a characteristic UV absorption at 345 nm dependent on the number of thioated nucleobases present.[126]

5-Hydroxymethyl-2'-deoxyuridine has demonstrated anti-leukemic and antiviral activities.[127] The insertion of this nucleoside in DNA may provide a better approach for studying its biological function. Conte *et al.*[128] described the preparation of the deoxyribonucleoside phosphoramidite **102** from 2'-deoxyuridine and its double and consecutive incorporation into Dickerson's dodecamer. The coupling efficiency of **102** was similar to that of the unmodified deoxyribonucleoside phosphoramidites. The dodecamer was characterized by ^{1}H-NMR spectroscopy and by enzymatic digestion with snake venom phosphodiesterase.[128] Further studies concerning the structure and properties of the modified oligomer are in progress.



102

3. INHIBITION OF GENE EXPRESSION

3.1. Oligonucleoside Phosphorothioates.

In the late seventies Burgers and Eckstein reported the synthesis of dinucleoside monophosphorothioates by the sulfurization of phosphite triesters with elemental sulfur.[129] Unlike dinucleoside monophosphates, the thiophosphate derivatives, being chiral at phosphorus, existed as pairs of diastereoisomers. Phosphorothioate analogues of ATP were particularly useful in the elucidation of the stereochemical course of enzymatic phosphoryl and nucleotidyl transfer reactions.[130] The chemical synthesis of dinucleoside phosphorothioates was easily accomplished by the phosphite triester approach[129,131] and by the phosphoramidite methodology.[130b,132] The configuration of the chromatographically resolved diastereoisomers was assigned by hydrolysis with snake venom phosphodiesterase (SVP) and nuclease P1 which exclusively cleaved the *Rp* or the *Sp* diastereoisomer, respectively.[130b] The incorporation of a phosphorothioate diester function into an octanucleotide (d[pGGpsAATTCC]) containing the *Eco*RI recognition sequence (GAATTC) showed that only the *Rp* diastereoisomer was recognized and cleaved, albeit slowly, by the endonuclease.[133a,b] The *Sp* diastereoisomer was not a substrate for *Eco*RI.[133b,134] Others[135a-o] have pursued this pioneering work and a wealth of information regarding the insertion of phosphorothioates,[135a-c,e,n,o] alkyl phosphonates,[135c,e,f,j] and alkyl phosphotriesters[135c-e,g-i,k,o] at specific locations throughout the *Eco*RI recognition sequence has been published in an attempt to rationalize the interactions of the endonuclease with the phosphate backbone of its recognition site. It has also been shown by Taylor *et al.*[136a] and others[136b] that many restriction enzymes did not cleave the DNA strand having a phosphorothioate diester link at the specified cleavage site but cleaved the unmodified complementary strand at the expected site.[136a-b] These data suggest that phosphorothioated DNA may find application in the sequence-specific preparation of nicked DNA duplexes.

Along similar lines, oligoribonucleotides containing a configurationally defined phosphorothioate function have been applied to probe the cleavage mechanism of hammerhead ribozymes.[137] It was found that, when located at the cleavage site, the *Rp* phosphorothioate isomer was cleaved very slowly in the presence of magnesium ion. In contrast, the cleavage of the *Sp* isomer was only slightly reduced from that of the natural phosphodiester. It has, therefore, been speculated that the magnesium ion was bound to the pro-*R* oxygen in the transition state of the hammerhead cleavage reaction.[137]

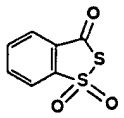
The incorporation of internucleotidic phosphorothioate linkages into oligonucleotides has considerably enhanced their resistance to hydrolysis by nucleases relative to unmodified oligonucleotides.[130a,134,138-143] As a consequence of this attribute, oligodeoxyribonucleoside phosphorothioates have demonstrated their usefulness as "antisense" molecules by inhibiting gene expression.[141c,144-154] In this context, the design, the synthesis, the application of oligodeoxyribonucleotides, oligoribonucleotides, and their analogues to the regulation of gene expression have been reviewed.[144a,d,151,155-163] For example, it has been demonstrated that antisense phosphorothioates complementary to the messenger RNA (the sense sequence) of the HIV-I *rev* gene, inhibited the cytopathic effect of the virus in chronically infected H9 cells.[164] The inhibition of HIV-LTR gene expression by oligonucleoside phosphorothioates targeted to the TAR element in a cell culture model has also been reported.[165] In addition, phosphorothioate oligomers exhibited antimalarial activities in strains of *Plasmodium falciparum*[166a] while antiviral activity against herpes simplex virus type 2,[166b-e] influenza A,[167] and influenza C[167] in cell cultures has also been recorded. Consequently, oligodeoxyribonucleoside phosphorothioates may represent a new class of experimental chemotherapeutic agents against AIDS,[168] hepatatis B,[169] and other infectious diseases.[141c,170,171a] The availability of these oligonucleotide analogues is therefore crucial for clinical investigations.[171]

The automated synthesis of oligonucleoside phosphorothioates can be performed according to the phosphoramidite approach.[142,144b,172a] The stepwise aqueous iodine oxidation responsible for the conversion of phosphite triesters to phosphotriesters was replaced with a relatively slow sulfurization reaction (7.5 min) requiring elemental sulfur (S_8).[142] Because of the poor solubility of S_8 in most organic solvents, the sulfurization reaction has been problematic and has often led to instrument failure. Four sulfur-transfer reagents were recently reported to eliminate the problems associated with the use

of elemental sulfur. Specifically, low concentrations (0.05 M-0.2 M) of the thiosulfonate **103** in acetonitrile converted a dinucleoside phosphite triester to the corresponding phosphorothioate dimer in yields better than 99% within 30 s at 20 °C.[173a-d] This reagent led to a rapid, efficient, and reliable automated synthesis of phosphorothioate oligomers carrying either exclusively or a predetermined number of P(S) links without detectable modification of the nucleobases.[141c,154,165,173a-c,e,174,175] The sulfur-transfer reagent **103** and its synthetic precursor, 3*H*-1,2-benzodithiol-3-one, have additionally been applied to the sulfurization of *H*-phosphonate and *H*-phosphonothioate diesters.[176]

Dibenzoyl tetrasulfide (**104**) has also demonstrated rapid sulfurization kinetics and satisfactory solubility properties during the solid-phase synthesis of a fully phosphorothioated nonadecadeoxyribonucleotide by the phosphoramidite approach. A solution of **104** (0.4 M) in tetrahydrofuran effected the sulfur-transfer reaction in near quantitative yields within 1 min.[177a] Phenylacetyl disulfide (**105**)[177b-c] and *N,N,N',N'*-tetraethylthiuram disulfide (**106**)[153b,178] were similarly applied to the solid-phase synthesis of oligodeoxyribonucleoside phosphorothioates. Relative to **103**, the sulfurization kinetics provided by **105** were sluggish. A 5% solution of phenylacetyl disulfide in 1,2-dichloroethane/*sym*-collidine (4:1) enabled the sulfurization of a dinucleoside phosphite triester within 5 min at 20 °C.[177b-c] Even slower sulfurization rates were obtained with **106** in acetonitrile as it took 15 min for the reagent (0.5 M) to completely sulfurize phosphite triesters.[178]

The synthesis of oligonucleoside phosphorothioates containing exclusively P(S) linkages can alternatively be accomplished by the sulfurization of *H*-phosphonate oligomers with elemental sulfur[145a,179,180] but the preparation of oligonucleotides having P(S) links at defined positions could not be easily achieved by this approach.



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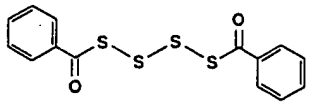
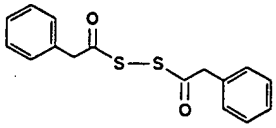
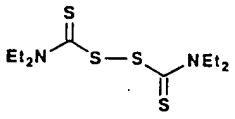


104



105

The efficiency of the sulfur-transfer reagent **103** has been further tested in the solid-phase synthesis of oligoribonucleoside phosphorothioates. Morvan *et al.*[181] described the preparation of such an oligoribonucleotide analogue from the ribonucleoside phosphoramidite **107**. The stepwise sulfurization reaction was complete within 30 s and the resulting oligoribonucleoside phosphorothioate exhibited enhanced stability to nucleases and satisfactory base-pairing abilities. These preliminary studies indicate that RNA phosphorothioates deserve consideration as potential antisense molecules.



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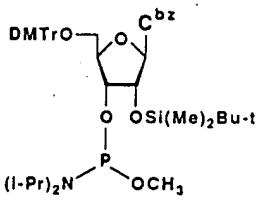
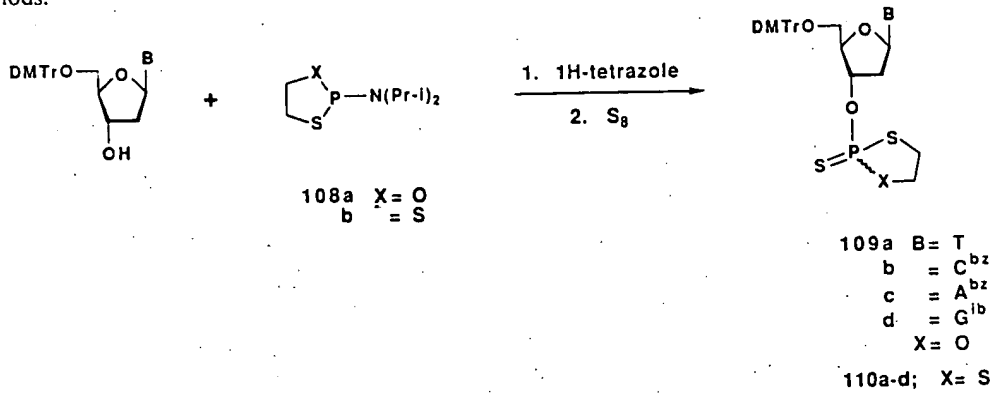


107

Gao *et al.*[174] reported that oligonucleoside phosphorothioates can inhibit human DNA polymerases and RNase H *in vitro*. It has been demonstrated that the phosphorothioate oligomer *S*-dC_{28} was a competitive inhibitor of DNA polymerase α and ß with respect to DNA template. *S*-dC_{28} was also a competitive inhibitor of RNase H_1 and H_2 with respect to RNA-DNA duplex. These inhibitory effects were not sequence-specific and depended on the total number of thioate linkages rather than on the position of the linkages within the oligomer or the chain length itself. To minimize such inhibitory effects, it has been recommended to decrease the number of phosphorothioate functions to *ca.* 15-20 per oligonucleotide.[174]

In addition to providing chirality to the phosphate backbone of oligonucleotides, the presence of the larger sulfur atom led to subtle conformational changes and altered the charge distribution around the phosphate group.[182] Furthermore, the configuration of the phosphorothioate function has been shown to have a striking influence on the B-Z transition of d(GC) and d(CG) octamers containing alternating phosphorothioate links.[183] There are also lines of evidence suggesting that internucleotidic phosphorothioate linkages may induce sequence-specific conformational changes in DNA that affect duplex stability.[184]

In this regard, a significant advance in the stereospecific synthesis of oligodeoxyribonucleoside phosphorothioates has recently been published.[185] The approach entailed the preparation of the nucleoside 3'-*O*-(2-thio-1,3,2-oxathiaphospholanes) **109a-d** from the reaction of suitably protected nucleosides with the phosphoramidite **108a** and 1*H*-tetrazole followed by oxidation with elemental sulfur. Individual diastereoisomeric **109a-d** were separated by silica gel chromatography and the purity of each stereoisomer was assessed by ^{31}P-NMR spectroscopy. The reaction of diastereoisomerically pure **109a** with solid-phase bound thymidine and DBU gave dTpsT in 95% yield with a stereoselectivity greater than 99%.[185] The application of this methodology to the stereospecific synthesis of larger oligodeoxyribonucleoside phosphorothioates required a LCAA-CPG support having the leader nucleoside anchored through a succinyl-sarcosyl linker[186] to survive prolonged contact with DBU. The solid-phase synthesis of octamers has been achieved using diastereomerically pure **109a-d**. ^{31}P-NMR spectroscopy indicated that the content of phosphates was less than 1%. The stereoregular oligodeoxyribonucleoside phosphorothioates were characterized by enzymatic and electrophoretic methods.[185]

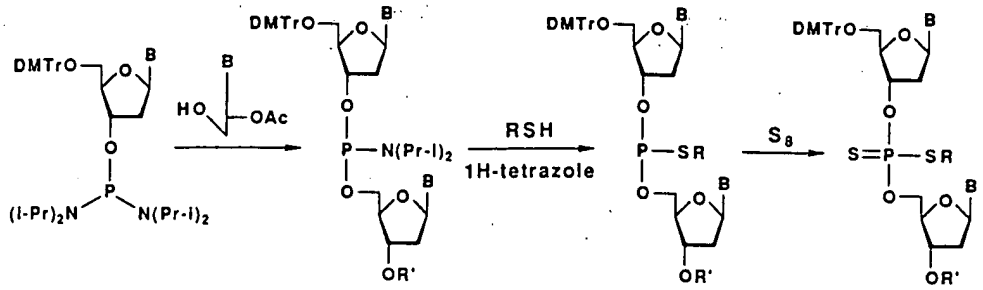


The stereospecific synthesis of oligodeoxyribonucleoside phosphorothioates should provide valuable structural informations regarding the interactions of these oligonucleotide analogues with target macromolecules. Such stereocontrolled syntheses should additionally shed light on the cellular uptake, distribution, and metabolism of these sulfur-containing oligonucleotides.

3.2. Oligonucleoside Phosphorodithioates.

The chirality at phosphorus in phosphorothioate oligonucleotides can be abolished by substituting sulfur for the remaining non-bridging oxygen atom of the internucleotidic phosphodiester function. The synthesis of dinucleoside phosphorodithioates has been delineated by Nielsen, Brill and Caruthers.[187a-c] Typically, the deoxyribonucleoside phosphorodiamidite **111** was converted to the dinucleoside phosphoramidite **113** upon reaction with 3'-*O*-acetylthymidine. Activation of **113** with 1*H*-tetrazole and hydrogen sulfide afforded the dinucleoside *H*-phosphonothioate **115** in 90% yield. The sulfurization of **115** with S_8 generated the desired dinucleoside phosphorodithioate **117** in 70% isolated yield.[187a] Replacing hydrogen sulfide with 4-chlorobenzyl mercaptan produced the dinucleoside thiophosphite **116** from **114**. In spite of the sensitivity of **116** to air oxidation, its sulfurization with elemental sulfur afforded **118** in 68% yield.[187d] The purified phosphorodithioate dimer was characterized by FAB mass spectrometry, ^{31}P- and ^{1}H-NMR spectroscopies. The dimer was resistant to digestion with SVP under

conditions which completely hydrolysed the natural dinucleoside monophosphate. It was also shown that the phosphorodithioate analogue of d(TpT) was resistant to the nucleolytic activity of bovine spleen phosphodiesterase and nuclease P1.[188a]



111 B= T
112 = C^{tol}

113 B= T; R'= Ac
114 B= C^{tol}; R'= Phac

115 B= T; R= H; R'= Ac
116 B= C^{tol}; R= p-ClBn; R'= Phac

117 B= T; R= H; R'= Ac
118 B= C^{tol}; R= p-ClBn; R'= Phac

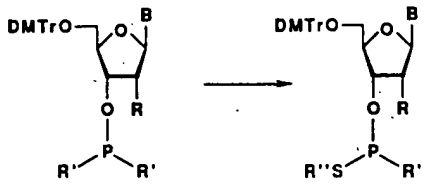
C^{tol}= N^4-(p-toluoyl)cytosin-1-yl; Phac= phenoxyacetyl; p-ClBn= p-chlorobenzyl

The preparation of oligodeoxyribonucleoside phosphorodithioates has alternatively been achieved *via* thioamidite intermediates.[187b,c,189] The preferred route for the synthesis of deoxyribonucleoside phosphorothioamidites involved the thiolysis of deoxyribonucleoside phosphorodiamidites with mercaptans.[189a,b,] Unexpectedly, the thioamidites **119** and **124** (Table 2) were relatively inert toward activation with 1*H*-tetrazole.[189a-b,190] It was rationalized that the steric bulk of the amidite *N*-substituents was significantly inhibiting the activation reaction.[189b,190] This rationale was found valid, as unlike **119** or **124**, the thioamidites **120-123**[189a] and **126**[189f] were readily activated with 1*H*-tetrazole and used in the solid-phase synthesis of oligodeoxyribonucleoside phosphorodithioates (20-mers). Each coupling step occurred with an efficiency of *ca.* 96-98%[189f] and was followed by oxidation with elemental sulfur in pyridine/carbon disulfide (1:1). The deprotected oligonucleotides were submitted to ^{31}P-NMR spectroscopy which revealed the presence of phosphorodithioate linkages at 113 ppm (D_2O).[189a] Of practical importance, reporter groups such as monobromobimane, 5-iodoacetamidofluorescein or 3-(2-iodoacetamido)-PROXYL have been inserted at specific sites in oligonucleotides having phosphorodithioate linkages[189k] in a manner similar to that reported for oligonucleoside phosphorothioates.[191]

Alternate routes to the synthesis of deoxyribonucleoside phosphorodithioates have recently been published. For example, the reaction of protected deoxyribonucleosides with 2-(*N,N*-diisopropylamino)-1,3,2-dithiaphospholane (**108b**) and 1*H*-tetrazole followed by oxidation with elemental sulfur, afforded the deoxyribonucleoside-3'-*O*-(2-thio-1,3,2-dithiaphospholanes) **110a-d** in 74-89% yield. The derivative **110b** led to the solid-phase synthesis of an oligodeoxycytidine phosphorodithioate (10-mer) in the presence of DBU with an average coupling yield of 96%.[192]

The conversion of the deoxyribonucleoside phosphoramidite **132** to the monomeric phosphorodithioate **133** has been applied to the large-scale preparation of phosphorodithioate oligomers *via* the phosphotriester approach.[189d-e,193] In addition, activated dithiophosphoric acid esters,[194] deoxyribonucleoside-*H*-phosphonothioates,[195] and deoxyribonucleoside-*H*-phosphonodithioates[188,196] have been employed as starting materials in the synthesis of oligonucleoside phosphorodithioates. Recent data showed that dithioated DNA was not digested by either snake venom phosphodiesterase,[187d] calf spleen phosphodiesterase,[187d] nuclease P1,[188a,b] the (3'→5')-exonuclease

Table 2. Deoxyribo- and Ribonucleoside Phosphorothioamidite Derivatives.



B= Protected Nucleobases

Compound	R	R'	R''	References
119	H—	—N(Pr-i)$_2$	Cl—C$_6$H$_3$—CH$_2$—	187b,c
120	H—	—N(CH$_3$)$_2$	Cl—C$_6$H$_3$—CH$_2$—	187c,189a
121	H—	—N(CH$_2$)$_4$	Cl—C$_6$H$_3$—CH$_2$—	187c,189a
122	H—	—N(CH$_3$)$_2$	Cl$_2$—C$_6$H$_2$—CH$_2$—	189a,e,190
123	H—	—N(CH$_2$)$_4$	Cl$_2$—C$_6$H$_2$—CH$_2$—	189a,d,193b
124	H—	—N(Pr-i)$_2$	NCCH$_2$CH$_2$—	189b,190
125	H—	—NEt$_2$	NCCH$_2$CH$_2$—	189b
126	H—	—N(CH$_3$)$_2$	NCCH$_2$CH$_2$—	189b,f,190
127	H—	—NEt$_2$	Cl$_2$—C$_6$H$_2$—CH$_2$—	189b
128	H—	—N(Pr-i)$_2$	CH$_3$—	189c
129	t-Bu(Me)$_2$SiO—	—N(CH$_3$)$_2$	NCCH$_2$CH$_2$—	198
130	t-Bu(Me)$_2$SiO—	—N(CH$_3$)$_2$	Cl$_2$—C$_6$H$_2$—CH$_2$—	198
131	t-Bu(Me)$_2$SiO—	—N(CH$_2$)$_4$	Cl$_2$—C$_6$H$_2$—CH$_2$—	198

erivatives.

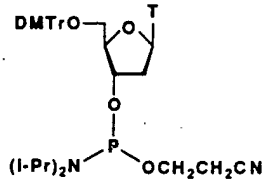
activity of bacteriophage T4 DNA polymerase, or by the nucleases present in HeLa cell nuclear extracts.[189d,e] The hybridization of a fully dithioated DNA segment with a complementary unmodified DNA sequence resulted in the formation of an hybrid having a lower T*m* relative to an unmodified DNA duplex.[197] The reduction of 0.5-2 °C per dithioate linkage was found to be higher than that observed with monothioated DNA (ΔTm = 0.4-0.6 °C per monothioate linkage).[189f] Like the phosphorothioate homooligomer ß-*S*-$(dC)_{28}$;[172a-c] oligodeoxyribocytidine phosphorodithioates[172d] inhibited *de novo* infection of susceptible cells by HIV-1. These oligonucleotide analogues are very potent inhibitors of HIV-1 reverse transcriptase *in vitro*. An increasing inhibitory effect correlated with the number of internucleotidic phosphorodithioate linkages. Comparative experiments demonstrated that a deoxyribodeoxycytidine phosphorodithioate oligomer (14-mer) was as effective as a phosphorothioate oligomer (28-mer) at inhibiting *de novo* infection by HIV-1.[172d]

References

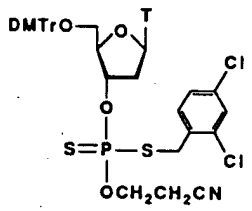
187b,c

187c,189a

187c,189a



132



133

189a,e,190

189a,d,193b

189b,190

189b

189b,f,190

189b

189c

198

198

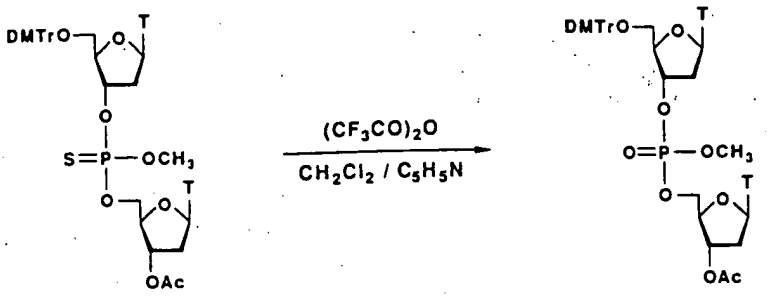
198

The synthesis of ribonucleotide dimers having internucleotidic phosphorodithioate linkages has been described by Petersen and Nielsen.[198] Similar to the preparation of deoxyribonucleoside phosphorothioamidites, the ribonucleoside phosphorothioamidites **129-131** (Table 2) were prepared from the thiolysis of the corresponding ribonucleoside phosphorodiamidites. The activation of **129** with 1*H*-tetrazole in the presence of a suitably protected ribonucleoside afforded, after sulfurization, the fully protected dinucleoside phosphorodithioate. The deprotected ribonucleotide analogue was resistant to hydrolysis with SVP and RNase A under conditions which totally hydrolysed the natural ribonucleotide. Moreover, the phosphorodithioate dimer was found virtually unchanged after treatment with concentrated ammonium hydroxide for 16 h at 5 °C. Collectively, the enhanced chemical stability of ribonucleoside phosphorodithioates and their resistance to nucleases should facilitate the study of biological processes involving RNA.[198]

Although the increased resistance of oligodeoxyribonucleoside phosphorothioates and dithioates to nucleases has been a desirable feature with respect to their application as potential therapeutics, it has nonetheless complicated the characterization of these oligonucleotides. To circumvent this problem, Burgers and Eckstein reported the desulfurization of an oligonucleotide containing internucleotidic phosphorothioate links with an excess of ethanolic iodine at 0 °C.[199] The desulfurized oligomer was then digested with SVP and bacterial alkaline phosphatase to the corresponding nucleosides. While aqueous iodine in pyridine[133b-c,183] or in 2,6-lutidine[133c] has also been applied to the desulfurization of oligodeoxyribonucleoside phosphorothioates, aqueous iodine in tetrahydrofuran/water/1-methylimidazole (16:3:1) led to the complete desulfurization of phosphorodithioated dimers and trimers.[188b] However, when applied to the desulfurization of a fully phosphorodithioated decanucleotide, these conditions produced extensive chain cleavage.[189f] In contrast, an aqueous solution of sodium metaperiodate has been effective in rapidly and cleanly desulfurizing oligonucleoside phosphorothioates (up to 20-mers).[133d]

An alternate approach to the desulfurization of thioated oligonucleotides may become available, as it has recently been reported that trifluoroacetic anhydride in pyridine effected the quantitative desulfurization of the dinucleoside phosphorothioate **134** to **135** within 24 h.[200] The desulfurization of model dithiophosphates such as $(RO)_2P(S)SR'$ under similar conditions yielded exclusively the corresponding monothiophosphates $(RO)_2P(O)SR'$.[200] This protocol may therefore be practically limited to the desulfurization of oligonucleoside phosphorothioates. It must, however, be noted that

dinucleoside phosphorodithioates have been converted to their corresponding dinucleoside monophosphates by treatment with butylene 1,2-oxide in ethanol-water for 120 min at 50 °C.[192]

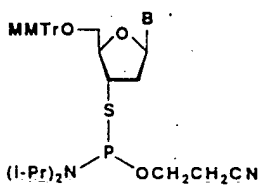


134 135

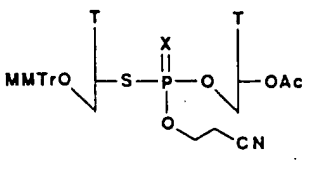
3.3. Oligodeoxyribonucleoside Phosphorothioates Achiral at Phosphorus.

Another class of sulfur-containing oligonucleotides emerged from the replacement of the bridging 3'-oxygen atom of the phosphodiester linkage with a sulfur atom.[201,202] The 3'-thio-2'-deoxyribonucleoside phosphoramidite **136a** was prepared from 5'-*O*-MMTr-3'-thiothymidine and chloro-(2-cyanoethoxy)-*N,N*-diisopropylaminophosphine.[201,202b] The insertion of **136a** in an oligothymidylate (d[TpTpTspTpT]) proceeded with a coupling efficiency of *ca.* 80% when 5-(*p*-nitrophenyl)-1*H*-tetrazole was used as an activator.[201] Because of the sensitivity of the P-S link to aqueous iodine, tetra-*n*-butylammonium oxone or, preferably, tetra-*n*-butylammonium periodate in dichloromethane was used as the oxidizing reagent in the automated synthetic cycle.[201,202b] The deprotected and purified oligomer was digested with nuclease P1 and alkaline phosphatase to thymidine and 3'-thiothymidine in a ratio of 4:1. Interestingly, the treatment of d(TpTpTspTpT) with 30 mM aqueous silver nitrate resulted in a clean and quantitative cleavage of the P-S linkage.[201]

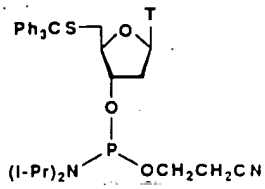
In addition to investigating the physical and chemical properties of a dithymidine phosphate analogue containing 3'-thiothymidine (**137**), Cosstick and Vyle[202b] reported the synthesis of dithymidine-3'-*S*-phosphorodithioate (**138**) from the condensation of **136a** with 3'-*O*-acetylthymidine followed by oxidation of the resulting dinucleoside thiophosphite triester with elemental sulfur. After deprotection and separation of the two diastereoisomeric dimers, the relative rates of enzymatic hydrolysis of d(TspT) and both *Rp* and *Sp*-d[Tsp(s)T] were determined. d(TspT) was digested with SVP as rapidly as the natural dinucleotide d(TpT). However, d(TpT) was hydrolysed 100 times faster than d(TspT) with nuclease P1. *Rp*-d[Tsp(s)T] was resistant to SVP and was hydrolysed 250 times slower than d(TpT) with nuclease P1. Conversely, *Sp*-d[Tsp(s)T] was not hydrolysed with nuclease P1 but was digested with SVP at 64% the rate observed for d(TpT).[202b] This work has been extended to d(AspT) which was synthesized from the reaction of the phosphoramidite **136b** with 3'-*O*-acetylthymidine and 5-(*p*-nitrophenyl)-1*H*-tetrazole.[202c] It should be emphasized that the fragility of the bridging phosphorothioate link to iodine or silver ions may become a useful tool for the site-specific "nicking" of DNA.[202d]



136a B= T
b = A^{bz}



137 X= O
138 = S

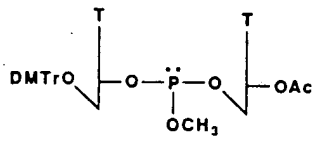


139

Recently, Mag *et al.*[203] delineated the synthesis of the 5'-tritylmercapto-2'-deoxyribonucleoside phosphoramidite **139** and its incorporation into a dodecamer by routine solid-phase synthesis. Oligo-5'-thiothymidylates[204] were sluggishly hydrolysed by both SVP and spleen phosphodiesterases.[204c,d] Typically, SVP hydrolysed dT_9 and $d(Tps)_8T$ to the extent of 50% and 10%, respectively, within 2 min.[205] Furthermore, the digestion of dT_{13} and $d(Tps)_{12}T$ with S1 nuclease proceeded with a half-life of 5 min and 350 min, respectively.[205] As expected from the data reported by Cosstick and Vyle[201] the internucleotidic link of these oligonucleoside phosphorothioate analogues was also chemically cleaved by 50 mM aqueous silver nitrate or mercuric chloride without affecting the natural phosphodiester linkages.[203]

3.4. "Boronated" Oligodeoxyribonucleotides.

The synthesis of an interesting class of oligonucleotide analogues having "boronated" internucleotidic linkages has been described by Sood *et al.*[206a] The synthetic approach consisted of the condensation of 5'-*O*-DMTr-2'-deoxythymidine-3'-*O*-(*N,N*-diisopropylamino) methoxyphosphine with 3'-*O*-acetylthymidine and 1*H*-tetrazole yielding the dinucleoside methyl phosphite triester **140** which upon reaction with borane-dimethylsulfide generated the dinucleoside boranophosphate methyl ester **141**. The purified dinucleotide analogue **141** was characterized by NMR spectroscopy (^1H, ^{13}C, ^{11}B, ^{31}P), FAB mass spectrometry, and elemental analysis. Incidentally, a ^{31}P-NMR spectrum of **141** revealed a broad peak at 118.0 ppm corresponding to the boranophosphate function.[206a] Treatment of **141** with concentrated ammonium hydroxide at ambient temperature or at 55 °C led only to the dinucleoside boranophosphate **142** without cleavage of the internucleotidic link. Shaking **141** with 1 M hydrochloric acid in methanol (1:1) at 20 °C resulted in a minimal conversion (less than 10%) of the boranophosphate methyl ester to the corresponding phosphate methyl ester according to ^{11}B- and ^{31}P-NMR spectroscopies.[206a,b] The boranophosphotriester function in **141** was also stable to the conditions required for chain extension by the phosphoramidite approach. The synthesis of a trimer having two boranophosphate triesters has been reported.[206a,b] Finally, it has been demonstrated that the dinucleoside boranophosphate **142** was quite resistant to the nucleolytic activity of both SVP and calf spleen phosphodiesterase (CSP). For example, the dinucleotide analogue **142** exhibited only 8% hydrolysis under conditions causing the hydrolysis of the natural dithymidylate to greater than 97%.[206a,b] Given the stability of internucleotidic boronated bridges to basic or acidic conditions and to exonucleases, oligonucleotides with boronated phosphates should further be investigated as potential therapeutic agents.



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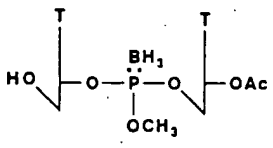

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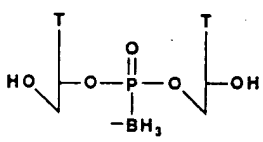
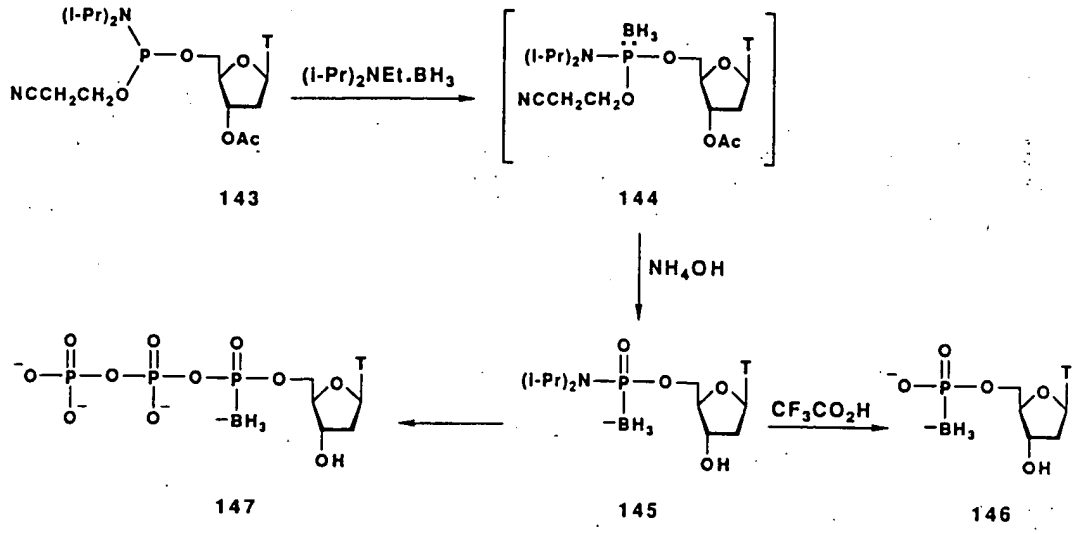


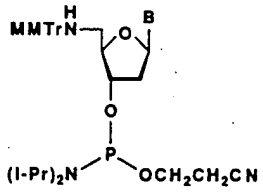
142

Of interest, the reaction of the deoxyribonucleoside phosphoramidite **143** with *N,N*-diisopropylethylamine-borane complex gave the intermediate **144** which upon treatment with ammonium hydroxide yielded the boranophosphoramidate **145**. Acid hydrolysis of **145** generated the novel thymidine 5'-boranophosphate **146**, whereas the reaction of **145** with bis-(tri-*n*-butylammonium)pyrophosphate yielded **147**.[206c] The thymidine 5'-boranophosphate **146** was hydrolyzed by snake-venom phosphodiesterase but was a poor substrate for *E. coli* alkaline phosphatase. The thymidine 5'-triphosphate analogue **147** can substitute for natural thymidine 5'-triphosphate in the complete extension of an heptadecaoligodeoxyribonucleotide primer using Sequenase (a modified T7 DNA polymerase) and a template (25-mer) containing one deoxyadenosine residue.[206c]



3.5. Oligodeoxyribonucleoside Phosphoramidates.

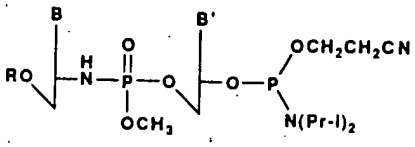
The properties of oligodeoxyribonucleotides having the bridging 5'-oxygen atom of the phosphodiester function replaced with a NH function have also been scrutinized. Although the synthesis of such achiral oligodeoxyribonucleoside phosphoramidates has been described earlier,[207] Mag,[208] Engels,[208] and Bannwarth[209] reported the preparation of the 5'-protected amino-2',5'-dideoxyribonucleoside phosphoramidites **148a-d** for the solid-phase synthesis of these oligonucleotide analogues. Particularly, the phosphoramidite **148c** was inserted in oligonucleotides at defined locations with an average coupling yield of 98-99%. The phosphoramidate link was stable to the acidic conditions required to remove the MMTr group but was completely cleaved upon incubation with 80% acetic acid for 5 h at ambient temperature.[208b,209] The primary amino function generated from this cleavage can be detected with great sensitivity and can thereby be used for diagnostic purposes.



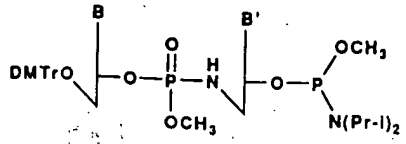
148a B= T
b = C^{bz}
c = A^{bz}
d = G^{Ib}

The phosphoramidite dimers **149a**[208c] and **150**[210] have also been synthesized to enable the incorporation of achiral phosphoramidate linkages at selected positions into oligonucleotides. Earlier studies indicated that oligonucleoside phosphoramidates having a 5'-terminal amino group were slowly hydrolysed by spleen phosphodiesterase under conditions which completely hydrolysed unmodified oligonucleotides.[207b] Phosphoramidate oligomers hybridized well to natural complementary sequences and acted as primers and templates in enzymatic reactions.[211a,b] Moreover, the multiple incorporation of the dinucleotidic phosphoramidite **149b** into oligothymidylates led to the formation of unusually stable complexes with poly (dA).[211c] Thus, these achiral oligonucleotide analogues deserve consideration as potential antisense molecules in the regulation of gene expression.

The synthesis of chiral oligonucleoside-*N*-alkylphosphoramidates has been described by Jäger *et al.*[212] Protected deoxyribonucleoside 3'-*O*-methyl-(*N,N*-diisopropyl)phosphoramidites were employed as building blocks in both solution and solid-phase oligonucleotide synthesis. The site-specific incorporation of an alkylphosphoramidate link was accomplished by replacing the usual aqueous iodine



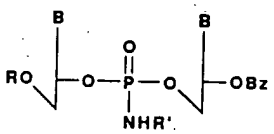
149a R= DMTr; B= T; B'= A^{bz}
b = Tr; B= B'= T



150 B= T, C^{bz}, A^{bz} or G^{ib}
B'= T or C^{bz}

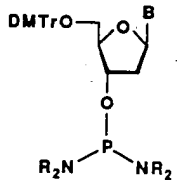
oxidation step with an iodine-alkylamine treatment. The phosphoramidate linkages in **151a-c** were resistant to the acidic conditions required for complete detritylation. In addition, a purified dinucleoside *N*-alkylphosphoramidate obtained from **151b** was resistant to digestion with nuclease P1 under conditions that would quantitatively hydrolyse d(TpT).[212] In this context, the synthesis of chiral (3'→5')-dithymidylyl phosphoranilidates from the reaction of a solid-phase bound (3'→5')-dithymidylyl methyl phosphite triester with iodine/aniline/2,6-lutidine has been reported.[135e] Following deprotection, the diastereoisomeric phosphoranilidates were separated and were shown to resist SVP-catalyzed hydrolysis.[135e]

The dinucleoside phosphoramidates derived from **152a-c** hybridized to poly-thymidylic acid and the T*m* of the resulting duplexes increased with the chain length of the *N*-alkylphosphoramidate substituent.[212] The insertion of phosphoramidate links in oligonucleotides has also been achieved *via* the deoxyribonucleoside phosphorodiamidites **153a-d** or **154a-d**.[213a-c] Typically, **153a** was coupled, in the presence of 5-(*p*-nitrophenyl)-1-*H*-tetrazole, to a trinucleotide prepared from ß-cyanoethyl deoxyribonucleoside phosphoramidites. A non-aqueous oxidation with *tert*-butyl hydroperoxide generated a 5'-terminal *N,N*-diethylphosphoramidate function resisting to the nucleolytic activity of SVP.[213a] Alternatively, the *N,N*-dialkylphosphoramidite or *N*-morpholinophosphoramidite linkages incorporated into oligonucleotides were hydrolysed under mildly acidic conditions to the corresponding *H*-phosphonate diesters[213a,c,214] which can potentially be converted to a variety of *P*-chiral analogues or natural phosphodiesters.[179]



151a R= MMTr; R'= n-C_4H_9; B= T
b = n-C_8H_{17}
c = n-$C_{12}H_{25}$

152a-c R= DMTr; R'= n-C_4H_9; B= A^{bz}
= n-C_8H_{17}
= n-$C_{12}H_{25}$



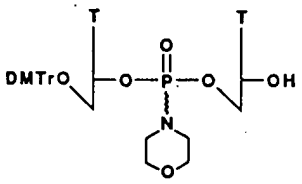
153a B= T; R= Et
b = C^{bz}
c = A^{bz}
d = G^{ib}

154a-d NR_2= morpholino

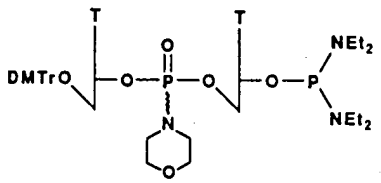
Of particular interest, Shimidzu *et al.*[215] reported the synthesis and properties of oligothymidylate analogues having alternating phosphodiester and stereodefined phosphormorpholidate linkages. These oligomers were prepared by inserting the dimeric bis-amidites **156a-b** in oligothymidylates. A coupling efficiency of *ca.* 89% was obtained when 5-(*p*-nitrophenyl)-1*H*-tetrazole was used as an activator. The internucleosidic phosphoramidite linkages were hydrolysed to *H*-phosphonate diesters upon treatment with 1*H*-tetrazole and water.[215a] Incidentally, **156a-b** were prepared from the phosphitylation of the diastereomerically pure dithymidylyl phosphormorpholidate derivatives **155a-b**[216a] with bis-(*N,N*-diethylamino)chlorophosphine. Incubation of the undecathymidylate analogues *Rp* and *Sp*-

d([$Tp_{morpholinyl}$Tp]$_5$T) with S1 nuclease resulted in the cleavage of a 5'-thymidine monophosphate at the 3'-terminus of the oligomers. The remaining d([$Tp_{morpholinyl}$Tp]$_4$$Tp_{morpholinyl}$T) was not hydrolysed further by the enzyme. Interestingly, the rate at which 5'-thymidine monophosphate was cleaved from the *Rp*-undecathymidylate was faster than that of the *Sp*-oligothymidylate. These data indicated that S1 nuclease recognized differences in configuration around the 3'-terminus of these oligonucleotide analogues.[215a] Phosphodiesterase I digested the above undecathymidylate analogues to 5'-thymidine monophosphate, dithymidylyl phosphormorpholidate, and 5'-phosphorylated dithymidylyl phosphormorpholidate. The *Rp*-oligomer was also digested faster than the *Sp*-oligomer with this enzyme. In contrast, micrococcal nuclease hydrolysed the oligothymidylate analogues at a much slower rate than that observed with phosphodiesterase I and, in this case, the *Sp*-oligothymidylate was digested faster than the *Rp*-congener.[215a]

Hybridization studies showed that the *Sp*-oligothymidylate analogue formed an hybrid with poly dA having a T*m* of 28 °C which compared well with that of the native oligothymidylate dT_{11} under similar conditions (T*m* = 30 °C).[215a,d] These results indicate that the *Sp* oligothymidylate analogue had a favorable configuration around the phosphormorpholidate linkage during complex formation. In this context, the *Rp*-oligothymidylate analogue did not form a stable hybrid with poly dA. Thus, the incorporation of stereoregulated phosphormorpholidate linkages into oligonucleotides represents an effective way of enhancing the potential of these analogues as regulatory molecules in the control of gene expression.[215a]



155a ***Rp*** **isomer**
b ***Sp*** **isomer**

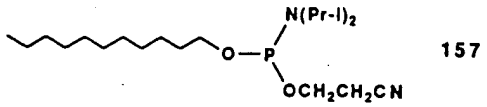


156a-b

Based on Letsinger's original procedure for rapid synthesis of oligodeoxyribonucleotides,[217] dichloro-(*N,N*-diethylamino)phosphine has been applied to the synthesis of oligonucleoside phosphoramidates.[218] However, chiral oligodeoxyribonucleoside phosphoramidates have predominantly been prepared by the *H*-phosphonate approach,[145a-b,179,219-222a] the nucleophilic substitution of internucleotidic phosphate triesters by alkylamines,[223] the activation of internucleotidic phosphodiesters with triphenyl phosphine and carbon tetrachloride,[224] the addition of alkyl azide to internucleotidic phosphite triesters[225] or by the oxidation of the latter with iodine in the presence of alkylamines.[226] Like oligodeoxyribonucleoside phosphorothioates, phosphoramidate analogues such as phosphormorpholidates, phosphorbutylamidates, and phosphorpiperazidates have exhibited anti-HIV activity in cell cultures.[145a-b]

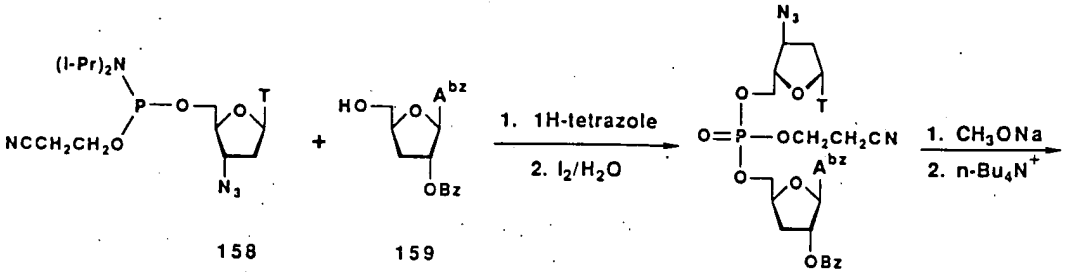
3.6. Hydrophobic Oligonucleotides and Oligodeoxyribonucleoside Methylphosphonates.

The application of oligonucleotides as potential therapeutic agents has been hampered by the low efficiency with which these molecules permeated intact cells. To increase the therapeutic potency of oligonucleotides, Kabanov *et al.*[227a] synthesized the phosphoramidite **157** as a means to introduce an hydrophobic group at the 5'-end of oligonucleotides with a coupling efficiency of 90-95%. Targeting an undecylated oligonucleotide against a loop forming-site of the influenza A/PR8/34 viral RNA considerably suppressed the development of the virus in permissive MDCK cells relative to that observed with unmodified oligonucleotides under identical conditions.[227a-b] The attachment of hydrophobic groups to oligonucleotides has therefore shown its efficacy at improving the biological activity of theses biomolecules.

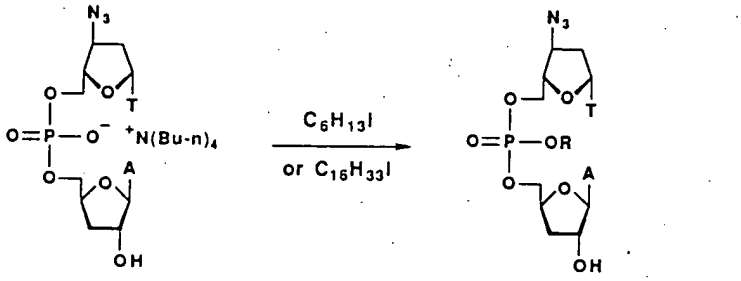


157

In an effort to synthesize lipophilic dinucleoside phosphate triesters as potential antiviral and antibiotic prodrugs, Meier and Huynh-Dinh[228] reported the preparation of the AZT phosphoramidite derivative **158**. Without isolation, **158** was reacted with N^6-benzoyl-2'-*O*-benzoyl-3'-deoxyadenosine (**159**) and 1*H*-tetrazole. Following aqueous iodine oxidation, the resulting (5'→5')-dinucleoside phosphotriester **160** was isolated by chromatography, deprotected, and ion-exchanged to the corresponding phosphodiester **161**. Alkylation of **161** with either 1-iodohexane or 1-iodohexadecane afforded the lipophilic phosphotriesters **162a-b** in 60% yield. These dimers were characterized by ^{1}H-, ^{13}C-, ^{31}P-NMR spectroscopies and by FAB-mass spectrometry.[228] The biological activity of **162a-b** against HSV-1 and HIV is being evaluated.



158 159 160



161

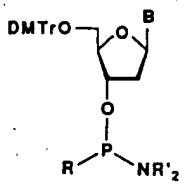
162a R= C_6H_{13}
b = $C_{16}H_{33}$

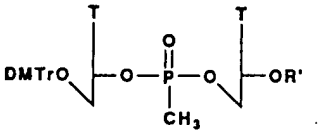
Nonionic oligomers such as oligodeoxyribonucleoside methylphosphonates are taken up by cells through passive diffusion[229] or, more likely, by a fluid phase/adsorbtive endocytic route.[230,231] This cellular uptake pathway is distinct from that of oligodeoxyribonucleoside phosphodiesters or phosphorothioates which seem to enter cells by endocytosis involving a saturable binding site.[230-232] Oligodeoxyribonucleoside methylphosphonates are totally resistant to nuclease hydrolysis[229] both in culture media and in cells.[231] By virtue of such attributes, these oligonucleotide analogues exemplify another category of antisense molecules with potential chemotherapeutic value as inhibitors of gene expression.[233]

The basic concepts stemming from the phosphoramidite approach led Dorman *et al.*[234] to the synthesis of the deoxyribonucleoside methyl phosphonamidite **163a**. Specifically, the reaction of 5'-*O*-DMTr-2'-deoxythymidine with chloro-(*N,N*-dimethylamino)methylphosphine or *N,N*-diisopropylamino-(*p*-nitrophenyloxy)methylphosphine and sodium hydride[235] afforded **163a** and **164a** respectively, in 95% isolated yield. Unexpectedly, the activation of **163a** with 1*H*-tetrazole generated several products as judged by ^{31}P-NMR spectroscopy. Nonetheless, the activation of **163a** with imidazole in the presence of

3'-*O*-DMTr-2'-deoxythymidine followed by routine iodine oxidation afforded the expected dinucleoside methylphosphonate **165a** in *ca.* 90% yield.[234] Jäger and Engels[236] independently reported the preparation of **163b** from the reaction of a suitably protected deoxyribonucleoside with bis-(*N,N*-dimethylamino)methylphosphine and catalytic amounts of 2,4,6-collidine hydrochloride. It was further demonstrated that deoxyribonucleoside methylphosphonamidites reacted with 3'-*O*-benzoylthymidine within 1 min upon activation with 1*H*-benzotriazole. The resulting dinucleoside methylphosphonites were oxidized with *tert*-butyl hydroperoxide affording the corresponding dinucleoside methylphosphonates in 81% yield. Consistent with the observations of Dorman *et al.*,[234] the use of 1*H*-tetrazole for the activation of deoxyribonucleoside methylphosphonamidites induced the disproportion of (3'→5')-dinucleoside methylphosphonite intermediates to symmetrical (3'→3')- and (5'→5')-dinucleoside methylphosphonites.[236] Paradoxically, 1*H*-tetrazole has been used for the activation of the phosphonamidites **164a-d** in the solid-phase synthesis of oligodeoxyribonucleoside methylphosphonates having contiguous[145b,222a,b,231,237a-g] or non-contiguous[222a,237d-f,h-j] methylphosphonate linkages. The incorporation of the methylphosphonamidites occurred within 30-120 s with a coupling yield of 96-97%,[237a,b] without apparent formation of side products.[237i] The removal of the base protecting groups was effected with ethylenediamine/ethanol (1:1) at ambient temperature. Under these conditions *ca.* 1% of each internucleotidic phosphonate linkage was cleaved.[237i]

In the same context, Roelen *et al.*[238] have reported the preparation of the deoxyribonucleoside phosphonamidites **164a** and **164e-f** from the reaction of 5'-*O*-DMTr-2'-deoxythymidine with the appropriate bis(*N,N*-diisopropylamino)alkylphosphine in the presence of 2,4,6-collidine hydrochloride. The phosphonamidites **164a,e,f** were incorporated at a defined location into oligonucleotides by solid-phase synthesis using 1*H*-tetrazole as an activator. The coupling efficiency of **164a,e,f** was *ca.* 97%. The resulting phosphonite function was oxidized or sulfurized to the corresponding phosphonate or phosphonothioate internucleotidic linkage.[238]





163a B= T; R= R'= Me
b = C^{bz}
c = A^{bz}
d = G^{ib}

164a-d R= Me; R'= i-propyl
e B= T; R= n-butyl; R'= i-propyl
f = T; = n-octyl; = i-propyl

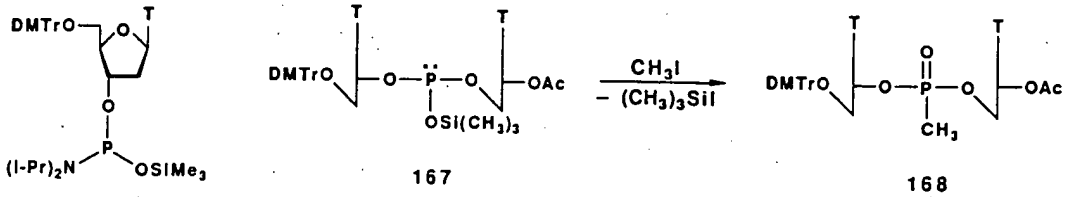
165a R'= DMTr
b = Ac

An interesting preparation of dinucleoside methylphosphonates has been described by Lebedev *et al.*[239a] Their approach is based on the reaction of the deoxyribonucleoside methylphosphonamidite 164a with 5'-*O*-trifluoroacetyl-3'-*O*-acetylthymidine and 4-(*N,N*-dimethylamino)pyridine (DMAP).[240] The coupling reaction proceeded to 73% yield within 2.5 h while, in the absence of DMAP, the coupling rate became *ca.* 100 times slower. This condensation reaction was less sensitive to trace amounts of water than those mediated by 1*H*-tetrazole.[239a] It must also be noted that the *Rp* and *Sp* diastereoisomers of the deoxyribonucleoside methylphosphonamidites **164a-d** can be separated on silica gel pretreated with triethylamine.[239b]

In a different application, the deoxyribonucleoside methylphosphonamidites **164a** and **164c-d** were incorporated at defined locations into an oligonucleotide containing the *Eco*RI recognition sequence. The *Rp* and *Sp* methylphosphonate diastereoisomers of each sequence were separated by HPLC. It was shown that *Rp*-*Rp* duplexes exhibited T*m* values similar to that of the parent unmodified duplex.

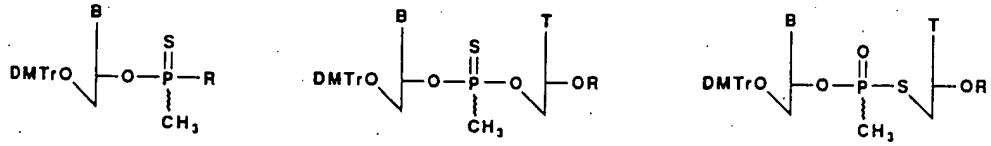
Conversely, *Sp*-*Sp* duplexes displayed lower T*m* values, probably because of the steric interactions imparted by the P-CH_3 group.[135j] In addition, methylphosphonate oligomers (larger than 8 bases) did not readily adopt a helical B geometry and hybridized poorly to natural DNA presumably because of conformational distorsions induced by the methyl group around the 3'- and 5'-*O*-*P*- linkages.[237c]

Of interest, Dabkowski *et al.*[241] reported the synthesis of the deoxyribonucleoside phosphoramidite **166** from 5'-*O*-DMTr-2'-deoxythymidine, bis-(*N,N*-diisopropylamino) trimethylsilyloxyphosphine, and *N,N*-diisopropylammonium tetrazolide. The condensation of **166** with 3'-*O*-acetylthymidine and *N,N*-diisopropylammonium tetrazolide afforded the dinucleoside trimethylsilyl phosphite triester **167** which upon treatment with methyl iodide yielded the dinucleoside methylphosphonotriester **168**.[241a] It must be pointed out that **167** could readily be hydrolysed to the corresponding *H*-phosphonate derivative[241a] or converted to either a dinucleoside phosphoroazolide[241a] or a dinucleoside phosphorofluoridate[242] which could all potentially be transformed into various *P*-chiral analogues.



166 **167** **168**

The synthesis of oligodeoxyribonucleoside methylphosphonothioates has also been investigated.[243] For example, the reaction of the phosphonamidite **164b** with hydrogen sulfide and 1*H*-tetrazole afforded the deoxyribonucleoside 3'-hydrogen methylphosphonothioate **169** in 94% yield. Treatment of **169** with iodine and 3'-*O*-acetylthymidine in pyridine generated the dinucleoside methylphosphonothioate **171** in 47% yield.[243a] Alternatively, the phosphonamidite **164a** was converted to the methylphosphonothioate **170** upon treatment with 3-hydroxypropionitrile, DMAP, and trifluoroacetic anhydride, followed by oxidation with elemental sulfur.[243c] After decyanoethylation of **170**, the lithium salt of each of the purified *Rp* and *Sp* diastereoisomers were condensed with 5'-deoxy-5'-iodo-3'-*O*-acetylthymidine affording the corresponding dinucleoside methylphosphonothioate **172**.[243c] Along similar lines, Brill and Caruthers[244] reported the synthesis of the phosphoramidite **173** and its congeners having any combination of the four nucleobases for the incorporation of stereospecific methylphosphonothioate linkages into synthetic oligomers.



169 B= C^{bz}; R= H
170 B= T; R= OCH_2CH_2CN

171 B= C^{bz}; R= Ac

172 B= T; R= Ac
173 B= T; R= $P[N(Pr\text{-}i)_2]OCH_2CH_2CN$

Of practical importance, the incorporation of two contiguous methylphosphonate linkages at each terminus of an oligonucleotide considerably increased its resistance to hydrolysis by exonucleases.[237f,i,245] Unlike natural oligodeoxyribonucleotides and their phosphorothioate analogues, oligodeoxyribonucleotides having exclusively or alternating methylphosphonate linkages were unable to form hybrids with RNA that were substrates for the RNase H activity of *E. coli*.[237f,246] However, methylphosphonate oligomers having three or more consecutive natural phosphodiesters elicited RNase H activity upon hybridization with complementary RNA.[237f,247,248] Thus, oligodeoxyribonucleoside

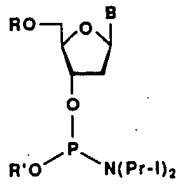
methylphosphonates with one or more regions having three successive phosphodiester functions provided a highly nuclease-resistant arrangement of methylphosphonate and phosphodiester linkages without loosing the ability to promote cleavage of the target RNA by RNase H.[237f,246,248] Oligodeoxyribonucleoside methylphosphonates have also demonstrated anti-HIV activity in infected cells.[145b,237h,249,250]

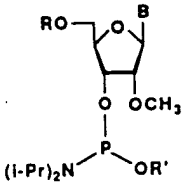
3.7. Oligonucleoside Phosphotriesters.

The phosphoramidites **174a-d** were applied to the synthesis of phosphate-methylated oligodeoxyribonucleotides to study the conformation of parallel mini-duplexes[251a-c] and to determine the stability of the hybrids formed between these oligonucleotide analogues and natural DNA or RNA.[251d] Buck *et al.*[251d,e] argued that phosphate-methylated oligonucleotides had an optimal combination of steric and stereoelectronic factors for the formation of the strongest hybrids with unmodified DNA.[251d,e] Such oligonucleotide analogues have been claimed to interrupt the life cycle of HIV-1, the causative agent of AIDS.[251f] Since then, this claim has been retracted.[251g] The notorious lability of methyl phosphotriesters required the development of procedures allowing the deprotection of the nucleobases without affecting the methyl phosphate protecting group. Kuijpers *et al.*[252] reported the synthesis of well-defined phosphate-methylated DNA segments from methyl and ß-cyanoethyl phosphoramidite derivatives of N^6-Fmoc-5'-*O*-DMTr-2'-deoxyadenosine and 5'-*O*-DMTr-2'-deoxythymidine, respectively. A 0.05 M solution of potassium carbonate in methanol was shown to remove both Fmoc and ß-cyanoethyl protecting groups while releasing oligomers from the solid support and leaving intact methyl phosphate protecting groups.[252]

The solid-phase synthesis of a phosphate-methylated oligonucleotide (d[Tp(OMe)]$_5$T) has also been achieved by Alul *et al.*[253] Using standard phosphoramidite chemistry on a Controlled-Pore Glass (CPG) support derivatized with an oxalyl linker, the formation of phosphodiesters was detected during the oxidation of methyl phosphite triesters with aqueous iodine. The search for an alternate oxidant led to a 0.5 M solution of *tert*-butyl hydroperoxide in dichloromethane which minimized unwanted demethylation. Upon completion of the synthesis, the phosphate-methylated oligomer was rapidly released from the support with 5% ammonium hydroxide in methanol. The oligomer was then isolated by HPLC and was characterized by FAB-mass spectrometry, migration on TLC, and by conversion to d([Tp]$_5$T) with thiophenol and triethylamine.[253]

Furthermore, the phosphoramidites **176a-b** have been applied to the synthesis of phosphate-methylated RNA dimers to instigate conformational analysis studies.[254]





174a-d R= MMTr; R'= Me
B= T, C^{fmoc}, A^{fmoc}, G^{fmoc}

175a-d R= DMTr; R'= Et
B= T, C^{bz}, A^{bz}, G^{ib}

fmoc= 9-fluorenylmethyloxycarbonyl

176a R= MMTr; R'= Me; B= C^{fmoc}
b = MMTr; = Me; = A^{fmoc}

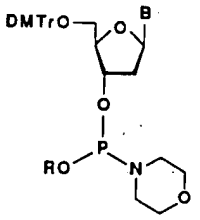
177a-d R= DMTr; R'= Me
178a-d = DMTr; = CH_2CH_2CN
B= U, C^{bz}, A^{bz}, G^{ib}

To evaluate the effectiveness of various oligonucleotide analogues as antisense molecules, the deoxyribonucleoside phosphoramidites **175a-d**,[135e,h] **179a-d**[135d] and the phosphonamidites **164a-b** were inserted at predetermined positions in oligonucleotides complementary to the translation initiation site of the mRNA encoding chloramphenicol acetyl transferase (CAT).[148] Oligonucleotides carrying ethyl phosphotriester functions led to 51% inhibition of plasmid-derived CAT gene expression in CV-1 cells.

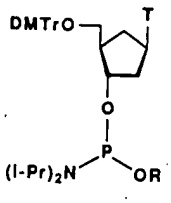
Under identical conditions, similar oligonucleotides having isopropyl phosphotriester, methylphosphonate, and natural phosphodiester linkages produced 0%, 65%, and 35% inhibition of CAT expression, respectively.[148] These findings suggest that the steric interactions created by either the ethyl or, most strikingly, by the isopropyl phosphate protecting group(s), may significantly affect the hybridization abilities of these oligonucleotide analogues with nucleic acid targets.

The deoxyribonucleoside phosphorodiamidites **153a-d** have particularly demonstrated their usefulness in the solid-phase synthesis of oligodeoxyribonucleoside isopropyl phosphotriesters.[255] Typically, **153a-d** were activated with 5-(*p*-nitrophenyl)-1*H*-tetrazole and incorporated at selected positions into oligonucleotides. A subsequent treatment with 5-(*p*-nitrophenyl)-1*H*-tetrazole in isopropyl alcohol:acetonitrile (1:4) followed by oxidation with *tert*-butyl hydroperoxide yielded each chiral isopropyl phosphotriester function with a coupling efficiency of *ca.* 99%. The *Rp* and *Sp* diastereoisomers of a tetradecamer were resolved by HPLC and it was shown that the *Sp* isomer was more potent than the *Rp* isomer in preventing primer chain elongation beyond the isopropyl phosphate protecting group.[255]

The deoxyribonucleoside phosphoramidite **180** has been employed in the synthesis of dinucleotide blocks toward the synthesis of octathymidylates having alternating neopentylphosphothionotriester/phosphodiester linkages of defined stereochemistry at phosphorus.[256] These oligothymidylate analogues were about 15 times more resistant to endonuclease P1 than unmodified oligonucleotides. No hybridization studies involving these oligonucleotides with complementary sequences were however presented.



179a-d B= T, C^{bz}, A^{bz}, G^{ib}
R= isopropyl
180 B= T; R= neopentyl



181 R= Me
182 = CH_2CH_2CN

The phosphoramidites **175a-d** were also applied to the solid-phase synthesis of the self-complementary DNA sequence d(GGAA[Et]TTCC). Upon separation of the diastereoisomers, it was demonstrated that the *Rp*-*Rp* duplex had a lower *Tm* than that of the *Sp*-*Sp* or the unmodified duplex (ΔTm = 11 °C).[135g] Similar modified DNA sequences were used to map the contact points between DNA and the endonuclease *Eco*RI.[135k,257,258]

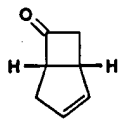
3.8. Oligonucleotides with Modified Carbohydrate Entities.

The search for novel synthetic oligonucleotides as potential inhibitors of gene expression has focused not only on the chemical modification of the atoms linked to the phosphate backbone (*vide supra*) but also on the chemical modification of the carbohydrate moieties. In this regard, the synthesis of carbocyclic oligothymidylates was initiated by the 1*H*-tetrazole-mediated coupling of the carbathymidine phosphoramidite **181** with (+)-carbathymidine (C-dT) linked to a solid support. C-dT_4 and C-dT_{12} were synthesized with a stepwise yield of *ca.* 90%. The stability of purified C-dT_4 and natural dT_4 to nucleolytic hydrolysis were compared. It was shown that calf spleen phosphodiesterase (CSP) hydrolyzed dT_4 *ca.* 9 times faster than C-dT_4. S1 nuclease did not hydrolyze C-dT_4 after 24 h of incubation, whereas dT_4 was 50% hydrolysed within 3.5 h under the same conditions. It was also found that C-dT_{12} formed a more stable hybrid with $d(C_2A_{12}C_2)$ relative to dT_{12} (ΔTm = 10 °C).[259]

Szemző *et al.*[260a] independently described the solid-phase synthesis of the carbocyclic

oligothymidylates C-dT_{10}, C-dT_{12} and C-dT_{20} using the phosphoramidite **182**. In agreement with the findings of Perbost *et al.*,[259] it was observed that the coupling efficiency of **182** was lower (95%) than that of the corresponding unmodified phosphoramidites (98%). Conformational and/or stereoelectronic differences between carbocyclic and natural nucleosides were invoked to explain the relative lack of reactivity of the phosphoramidite **182**. It has additionally been discovered that unlike dT_{10}, C-dT_{10} adopted a single-stranded helical structure in a solution of appropriate ionic strength.[260b] In spite of the stability of the duplex formed between a carbocyclic oligothymidylate and a complementary polynucleotide, the carbocyclic oligomer was inactive as a template or as a primer in DNA polymerase assays. It was postulated that $(C\text{-}dT)_n$ was strongly bound to the polymerase and thus inhibited the initiation of replication.[260b] The usefulness of carbocyclic oligomers as antisense molecules awaits further investigation.

Payne and Roberts[261] delineated the preparation of the (fluorocyclopentyl)thymine phosphoramidite **185** and its application to the synthesis of novel carbocyclic oligonucleotides. The precursor nucleoside **184** was obtained from the ketone **183** in 16% overall yield. The insertion of **185** in oligonucleotides proceeded with a coupling efficiency of 85% consistent with that observed with other carbocyclic phosphoramidites.[259,260a] Polyacrylamide gels provided evidence that fluorine-containing oligonucleotides can form hybrids with complementary strands. The susceptibility of these duplexes to cleavage by restriction enzymes is currently being studied. ^{19}F-NMR spectroscopy has been particularly helpful in detecting the presence of fluorine in these modified oligonucleotides.[261]



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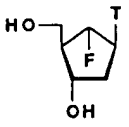

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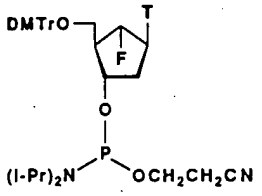

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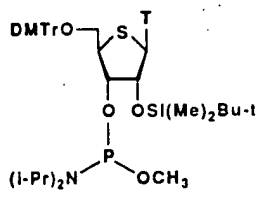


186

The synthesis of oligodeoxyribonucleotides containing 1-(4'-thio-ß-D-ribofuranosyl)thymine has recently been reported by Bellon *et al.*[262] The thioribofuranosyl phosphoramidite **186** was incorporated into dodecamers [$T^*(dT)_{11}$ and $(dT)_5T^*(dT)_6$, where T^* represents 1-(4'-thio-ß-D-ribofuranosyl)thymine] which were subjected to digestion with CSP and hybridization with complementary sequences. The 5'-exonuclease hydrolyzed $T^*(dT)_{11}$ and $(dT)_{12}$ to the extent of 50% within 43 min and 1 min, respectively, thus indicating the strong resistance of the modified oligonucleotide to the nucleolytic activity of CSP. The heteroduplex $d(C_2A_{12}C_2)/(dT)_5T^*(dT)_6$ and the parent duplex $d(C_2A_{12}C_2)/(dT)_{12}$ exhibited T*m* values of 40 °C and 45 °C, respectively.[262] 4-Thiooligonucleotides are being evaluated as antisense molecules inspite of their relatively low affinity for complementary sequences.

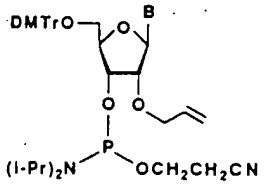
Chimeric oligonucleotides composed of deoxyribonucleotides and 2'-*O*-methylribonucleotides complementary to RNA fragments, induced site-specific cleavage of the RNA with *E. coli* RNase H.[263] The 2'-*O*-methylribonucleoside phosphoramidites **177a-d** and **178a-d** were used in the solid-phase synthesis of the ribonucleotidic part of the chimeric oligonucleotides, whereas standard deoxyribonucleoside phosphoramidites were utilized for the insertion of short DNA segments (3-5 bases) within the oligonucleotidic chains.[263a] The phosphoramidites **177a-d** and **178a-d** were also used to construct chimeric adapters containing double-stranded 2'-*O*-methyl RNA that would withstand the nucleolytic activity of Bal 31. It was shown that seven consecutive 2'-*O*-methyl ribonucleotide residues were sufficient to provide resistance to Bal 31 hydrolysis.[264] This feature allowed the unidirectional digestion of DNA with Bal 31. New synthetic routes to the synthesis of protected 2'-*O*-methyl- or 2'-*O*-ethylribonucleoside-3'-*O*-phosphoramidites have recently been developed by Wagner *et al.*[265] and Sproat *et al.*[266]

Oligomers composed of unmodified ribonucleotides and their 2'-*O*-methyl analogues have been prepared from the respective phosphoramidites to study RNA binding proteins and/or RNA processing enzymes.[267] The presence of 2'-*O*-methylribonucleotides conferred protection against nucleases and stability to alkaline hydrolysis. A highly efficient synthesis of these oligoribonucleotides on a urethane-linked aminopropylated CPG support using the phosphoramidites **178a-d** and 5-(*p*-nitrophenyl)-1*H*-tetrazole as an activator has been reported.[268] Given the steric hindrance caused by the neighbouring 2'-*O*-methyl group, a longer condensation time (6 min) was required to ensure high coupling efficiency (>99%). The enzymatic stability of 2'-*O*-methylribonucleotides were compared to identical DNA and RNA sequences under the same conditions. 2'-*O*-Methyl RNA oligomers were resistant to a variety of RNA and DNA nucleases but were sensitive to P1 nuclease, snake venom phosphodiesterase, and Bal 31 nuclease.[268] The sensitivity of single-stranded 2'-*O*-methyl RNA to Bal 31 is in sharp contrast with the remarkable stability of their double-stranded congeners to this nuclease.[264]

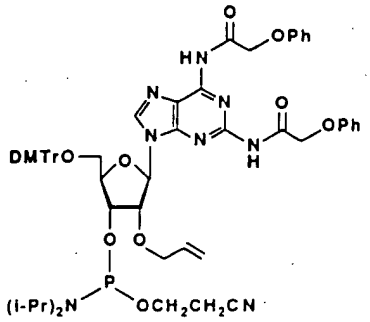
Of interest, the incorporation of N^4-benzoyl-5'-*O*-DMTr-2'-*O*-methylcytidine-3'-*O*-(*N,N*-diisopropylamino)methoxyphosphine (**177b**) at a defined location into an oligoribonucleotide confirmed the participation of the 2'-OH group of the substrate in a cleavage reaction catalysed by a synthetic ribosyme.[269a] Furthermore, 2'-*O*-methylribonucleotides prepared by the phosphoramidite approach were also used for probing the structure and function of U1,[270d] U2,[270a,c] U4,[270b,g] U5 and U6[270b,g] small nuclear ribonucleoprotein particles (snRNPs) which are subunits of functional spliceosomes.[270f]

The stability of heteroduplexes composed of 2'-*O*-methyl RNA and complementary RNA fragments[271] in addition to the decreased sensitivity of these analogues to several nucleases, have enhanced the suitability of these oligonucleotides as antisense molecules. Incidentally, 2'-*O*-methyl oligoribonucleotides have been designed to specifically bind to sequence flanking the HIV-1 *gag-pol* RNA hairpin in an attempt to alter −1 ribosomal frameshifting, and the expression of the *gag* and *pol* genes.[272] Oligonucleotides binding just downstream to the stem of the hairpin enhanced ribosomal frameshifting by 6-fold. Conversely, oligonucleotides binding upstream to the stem had no effect on frameshifting efficiency. The efficiency of −1 frameshifting of retroviral RNA determines the ratio of *gag-pol* gene products which is critical to viral assembly. Thus, inhibition or enhancement of ribosomal frameshifting may affect viral production and might disrupt the HIV life cycle.[272]

The preparation of the 2'-*O*-allyl-3'-*O*-phosphoramidites **187a-e** has been described by Sproat *et al.*[273a] and others.[273c] The solid-phase synthesis of oligoribonucleotides began with the activation of **187a-d** with 5-(*p*-nitrophenyl)-1*H*-tetrazole. The coupling time was extended to 8 min to ensure high condensation yields.[273a] It must be pointed out that RNA oligomers prepared from base-protected 5'-*O*-DMTr-2'-*O*-(3,3-dimethylallyl)ribonucleoside 3'-*O*-(ß-cyanoethoxy)-*N,N*-diisopropylaminophosphines were not as efficient as 2'-*O*-allylribonucleotides in providing a rapid and stable binding to complementary RNA sequences.[274] Consequently, 2'-*O*-allylribonucleotides may serve as antisense molecules in diverse areas of molecular biology.



187a B= uracil
b = N^4-benzoyl cytosin-1-yl
c = N^6-trimethylacetyl adenin-9-yl
d = N^2-dimethylformamidine guanin-9-yl
e = hypoxanthin-9-yl



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The synthesis of novel 2'-*O*-modified oligonucleotides from nucleoside phosphoramidite intermediates has also been reported by others.[275] Preliminary data regarding the biophysical and biological evaluation of these oligonucleotide analogues were also presented.

Lamm *et al.*[276a] reported the incorporation of the 2,6-diaminopurine-2'-*O*-allylribonucleoside-3'-*O*-phosphoramidite **188**[273a-b] into oligoribonucleotides in an attempt to specifically deplete U5 snRNP from HeLa cell nuclear splicing extracts. It was demonstrated that U5 snRNP was essential for spliceosome assembly. The absence of U5 snRNP prevented the stable association of U4/U6 but not that of U1 and U2 snRNPs with pre-mRNA.[276a] Biotinylated 2'-*O*-allyloligoribonucleotides having 2-aminoadenine nucleobases were also used to deplete U2 snRNA from HeLa nuclear extracts.[276b] These oligonucleotides were not significantly inhibiting spliceosome assembly because accumulation of splicing intermediates was observed. The modified oligonucleotides inhibited mRNA production *in vitro* by interfering with exon ligation. These results suggest that the functional requirement for U2 snRNP in the splicing mechanism occurred after spliceosome assembly.[276b]

Cotten *et al.*[277] described the synthesis of 2'-*O*-methyl- and 2'-*O*-ethylribonucleotides as inhibitors of the U7 snRNP-dependent mRNA processing event. These oligonucleotides were prepared by solid-phase techniques using appropriate (ß-cyanoethyl)-*N,N*-diisopropylphosphoramidites. An extended coupling reaction time (5 min) produced an average coupling efficiency of 98%. It was shown that 2'-*O*-ethylribonucleotides were *ca.* 5 times more effective than that of native antisense RNA molecules at inhibiting mRNA processing. Only a slight excess of inhibitor over target RNA was required for an 80% inhibition of the processing reaction.[277]

Of particular importance, Shibahara *et al.*[278] reported that oligo-(2'-*O*-methyl)ribonucleoside phosphorothioates inhibited the replication of the human immunodeficiency virus (HIV-1) in cultured MT-4 cells. Interestingly, ribonucleotides having five phosphorothioate linkages at the 3'- and 5'-termini exhibited almost as much anti-HIV activity as fully phosphorothioated oligomers. In contrast, both 2'-*O*-methylribo- and deoxyribonucleotides failed to inhibit the replication of the virus. These data indicated that fully thioated oligonucleotides were not necessary to demonstrate anti-HIV activity and that oligo-(2'-*O*-methyl)ribonucleotides were sensitive to the nucleases present in the cell line used for the study.[278] 2'-*O*-Methylribonucleoside phosphorothioate oligomers have also been useful in deciphering the mechanisms whereby the expression of Intercellular Adhesion Molecule 1 was inhibited by antisense oligonucleotides.[154] Finally, 2'-*O*-methylribonucleotides and their phosphorothioate analogues were effective in forming pseudo half-knot structures with the HIV TAR element. This element is a structural RNA that binds to *Tat*, a viral regulatory protein. The binding of *Tat* to TAR RNA is a critical step in the viral life cycle. Pseudo half-knot formation disrupts the structure of TAR in the region specifically recognized by the *Tat* protein and thus offers a new therapeutic target site for antisense oligonucleotides.[279]

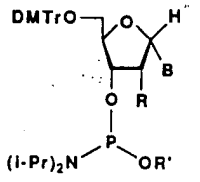
Almost two decades ago, dithymidylyl monophosphate composed of nucleosides having the sugar and the nucleobase moieties linked by an α-glycosidic bond, as opposed to the natural ß-glycosidic bond, was found to be a substrate for snake venom and bovine spleen phosphodiesterases.[280] The rate of enzymatic hydrolysis of α-dithymidylyl monophosphate was considerably lower than that of the corresponding ß-anomer.[280] This early observation led to the design of novel antisense molecules as potential therapeutics.[281]

Although the original preparation of α-oligodeoxyribonucleotides was accomplished by the phosphotriester methodology,[282a-c] the α-deoxyribonucleoside phosphoramidites **189a-d** and **190a-c** were prepared and applied to the solid-phase synthesis of α-oligonucleotides.[282c-e,283,284] Purified α- and ß-[d(CATGCG)] were subjected to hydrolysis with S1 nuclease, calf spleen phosphodiesterase (CSP), and snake venom phosphodiesterase (SVP).[282b] Under specific conditions, S1 nuclease and CSP hydrolysed the ß-oligomer to the extent of 100% and 89%, respectively, within 10 min. Under identical conditions, the α-oligomer was resistant to CSP and showed only 7% hydrolysis with S1 nuclease. SVP digested the α-oligomer at a rate 30 times slower than that of the ß-oligomer.[282b] In a similar study, Thuong *et al.*[285] pointed out that the stability of acridine-substituted α-oligothymidylates to CSP and SVP was roughly 300-500 times higher than that of similarly derivatized ß-oligothymidylates.

To evaluate the suitability of α-oligonucleotides as antisense molecules,[286] an α-

hexadecadeoxyribonucleotide was prepared from the phosphoramidites **189a-d**.[287] The half-life of the radiolabelled α-oligonucleotide in undiluted fetal bovine serum was *ca.* 24 h. In contrast, ß-oligomers were completely hydrolysed under these conditions within the first 15 min.[287] Furthermore, α-oligomers survived well in *Xenopus* oocytes with a half-life of over 8 h compared to that of only 10 min for ß-oligomers.[288] However, α-oligonucleotides exhibited poor stability in NIH3T3 cellular extracts. For example, an α-hexaadenylate was hydrolyzed to the extent of 50% within 132 min compared to that of 54 min for the corresponding ß-oligomer.[289]

NMR and UV absorption studies indicated that, unlike native DNA, α-oligodeoxyribonucleotides hybridized with complementary ß-strands in a parallel orientation.[282c,290] The hybrid composed of α-d(CATGCG) and ß-d(GTACGC) adopted a predominant right-handed B-conformation in aqueous solution.[290] The stability of the duplex depended on the base composition of the α-sequences; pyrimidine α-nucleosides provided more stability than purine α-nucleosides.[291] Interestingly, oligonucleotides composed of only α-thymidine have been shown to form antiparallel duplex-structures according to UV and CD spectroscopies.[292]



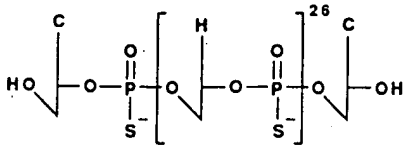
189a B= T; R= H; R'= Me
b = C^{bz}
c = A^{bz}
d = G^{pal} pal= palmitoyl
190a-c R= H; R'= CH_2CH_2CN
191a B= U; R= $OSi(Me)_2Bu\text{-}t$; R'= Me
b = C^{bz}
c = A^{bz}
d = G^{ac} ac= acetyl

α- and ß-Anomeric $d(G_2T_{12}G_2)$ oligodeoxyribonucleotides were prepared by the phosphoramidite approach and used in hybridization experiments with the riboadenylate rA_{12}. The ß-DNA/RNA and α-DNA/RNA hybrids exhibited a *Tm* of 27 °C and 53 °C, respectively.[284] The duplex ß-$d(G_2T_{12}G_2)/rA_{12}$ was a substrate for *E. coli* RNase H and resulted in the hydrolysis of rA_{12}. Conversely, α-$d(G_2T_{12}G_2)/rA_{12}$ did not lead to the degradation of rA_{12} with either *E. coli* RNase H or *Drosophila* embryo RNase H under identical conditions.[282c,284,293a] The parallel annealing of α-DNA to RNA was presumably responsible for the protection of RNA against RNase H-mediated hydrolysis.[293b] Such annealing may also account for the fact that the translation of rabbit ß-globin mRNA in cell-free systems and in micro-injected oocytes was not inhibited by an α-oligodeoxyribonucleotide targeted to the coding region of the message. However, the corresponding ß-oligodeoxyribonucleotides and ß-oligodeoxyribonucleoside phosphorothioates were inhibitory.[294] Paradoxically, α-oligodeoxyribonucleotides targeted against the 5'-cap region of rabbit ß-globin mRNA, specifically inhibited the translation of the message in rabbit reticulocyte lysates or wheat germ extracts.[295a] These experiments provided evidence that α-oligodeoxyribonucleotides can be potentially useful as inhibitors of translation. It has further been reported that an α-oligodeoxyribonucleotide (20-mer) targeted against the primer binding site of Friend murine leukemia virus, inhibited viral spreading.[295b] Moreover, Morvan *et al.*[173c] and Rayner *et al.*[296] described the solid-phase preparation of α-oligodeoxyribonucleoside phosphorothioates exhibiting anti-HIV activity. These included a dodecamer complementary to the splice acceptor site of the pre-mRNA encoding the *Tat* protein,[173e] and a 28-mer targeted against the translation initiation site of *rev* mRNA.[296]

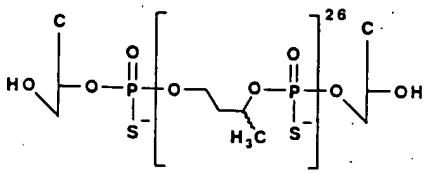
The synthesis of α-oligothymidylyl phosphorothioates on a teflon-based solid support has been accomplished *via* the α-deoxyribonucleoside phosphoramidite **189a** with coupling yields averaging 98%. The stepwise sulfurization reaction was effected by elemental sulfur or *N,N,N',N'*-tetraethylthiuram disulfide.[297a] Like standard oligodeoxyribonucleoside phosphorothioates, the α-oligothymidylyl phosphorothioates displayed superior resistance to nucleases relative to α-oligothymidylates.[297a] Furthermore, the oligothymidylyl phosphorothioate α-$d([Tps]_7Tp)$ formed a more stable duplex with polyriboadenylic acid than that observed with ß-$d([Tps]_7Tp)$ (ΔTm = 14 °C).[297b]

It is known that the phosphorothioate homooligomer ß-*S*-dC_{28} blocked *de novo* infection of susceptible cells by HIV-1.[172a-c] The α-*S*-dC_{28} oligomers displayed an anti-HIV activity comparable to

that of the ß-*S*-dC_{28} oligomer, whereas both ß-dC_{28} and α-dC_{28} were inactive.[296] These observations were expected since the abasic oligodeoxyribonucleoside phosphorothioate **192** and the oligomeric phosphorothioate **193** demonstrated significant anti-HIV activities as well.[298] These results and those from others[299] suggest that the anti-HIV activity of phosphorothioate homooligomers is not entirely related to the enhanced resistance of these oligonucleotides to nucleases.



192



193

The deoxyribonucleoside phosphoramidite **194** has been employed in the solid-phase synthesis of alternating α, ß-oligothymidylates with alternating (3'→3')- and (5'→5')-internucleotidic linkages (α, ß-dT_{28}).[300] These oligonucleotide analogues have been proposed as models for antisense oligodeoxyribonucleotides. The synthetic design was based on the assumption that oligonucleotides having alternating (3'→3')- and (5'→5')-internucleotidic phosphodiester links may not be as rapidly hydrolysed with nucleases as natural oligomers. However, this approach would reduce the number of hybridizing nucleobases by one half and, hence, seriously limit the application of these oligonucleotides as antisense molecules. It has been postulated that the alternate substitution of an α-monodeoxyribonucleotide for a ß-monodeoxyribonucleotide in the (3'→3')- and (5'→5')-internucleotidic linkage motif would minimize this limitation.[300]

The hybridization of the purified oligonucleotide analogues with complementary unmodified ß-dA_{28}, and polyriboadenylic acid (poly rA) led to hybrids having thermostability similar to that of duplexes composed of the ß-polythymidylyl phosphorothioate *S*-dT_{28} and ß-dA_{28} or poly rA. Furthermore, the α, ß-dT_{28} oligomer exhibited better resistance to the nucleolytic activity of SVP and CSP relative to that of unmodified oligothymidylates and α-oligothymidylates. Although α, ß-dT_{28} oligomers were less resistant than *S*-dT_{28} to SVP and CSP, the former oligonucleotide analogues were considerably more resistant to S1 nuclease.[300] These results indicate that alternating α, ß-oligothymidylates with alternating (3'→3')- and (5'→5')-internucleotidic phosphodiester linkages may represent a new class of antisense molecules achiral at phosphorus. Others[301,302] have also observed that oligonucleotides carrying terminal (3'→3')- and (5'→5')-internucleotidic phosphodiester linkages were stabilized against extracellular[301,302] and intracellular degradation.[302]

Debart *et al.*[303] reported the solid-phase synthesis of the α-anomeric uridylates α-rU_6, α-rU_{12}, and α-r(UCUUAACCCACA) from the α-ribonucleoside phosphoramidite **191a-c**. The duration of each condensation step was 15 min and resulted in an average coupling efficiency of 97%. Purified α-rU_6 was resistant to CSP, ribonuclease A, and S1 nuclease for at least 1 h, 35 min, and 5 h, respectively, at 37 °C. Conversely, ß-rU_6 was totally hydrolysed by ribonuclease A, and was digested to the extent of 86% with CSP and S1 nuclease under the same conditions. α-rU_6 was nonetheless 58% hydrolysed by SVP within 2 h at 37 °C. Under these conditions, SVP caused the near complete hydrolysis (94%) of ß-rU_6 within 2 min.[303a]

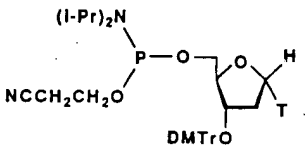
The dodecaribonucleotide α-r(UCUUAACCCACA) did not hybridize with ß-d(TGTGGGTTAAGA) above 0 °C. Nonetheless, this α-oligoribonucleotide led to the formation of a hybrid with ß-d(AGAATTGGGTGT) (Tm = 25.5 °C). Spectrophotometric evidence suggested the formation, at low temperature, of a triplex composed of two α-RNA and one ß-DNA strands.[303b] Interestingly, the α-dodecaribonucleotide being complementary, in parallel orientation, to the splice acceptor site of HIV-1 *tat* mRNA inhibited, with apparent lack of specificity, *de novo* HIV-1 infection of MT4 cells.[303b]

In contrast to α- and ß-D-oligodeoxyribonucleotides, very little is known about the properties of L-oligodeoxyribonucleotides. Morvan *et al.*[304] synthesized the ß-L- and α-L-deoxyribonucleoside phosphoramidites **195** and **200a** which were used in the solid-phase preparation of α- and ß-L-

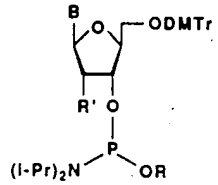
octathymidylates to evaluate the hybridization properties and stability of these oligonucleotide analogues to nuclease hydrolysis. Both α-L- and ß-L-octathymidylates were not digested by S1 nuclease or CSP within 24 h at 37 °C. Under identical conditions, a ß-D-octathymidylate was 50% hydrolysed within 69 min and 11 min, respectively, with these nucleases.[304] The annealing of either the α- or ß-L-octathymidylate with ß-D-octadeoxyriboadenylic acid (ß-D-dA_8) or poly rA failed at low ionic strength (0.1 M sodium chloride). Under these conditions, the natural ß-D-octathymidylate formed a duplex with ß-D-dA_8 (Tm = 5 °C) or poly rA (Tm = 10 °C).[304] It would, therefore, appear that α- or ß-L-oligodeoxyribonucleotides may not be effective antisense molecules.

The synthesis of oligodeoxyribonucleotides containing ß-L-2'-deoxyribose has also been reported by Damha *et al.*[305] Strategically, ß-L-2'-deoxyuridine was converted to ß-L-2'-deoxycytidine and both of these nucleosides were transformed into the phosphoramidite derivatives **196** and **197**. ß-L-Hexadeoxycytidylic and ß-L-hexadeoxyuridylic acids were prepared by standard solid-phase chemistry. In agreement with the observations of Morvan *et al.*,[304] ß-L-oligomers were completely resistant to the nucleolytic activity of CSP, P1 and S1 nucleases. ß-L-hexadeoxycytidylic acid and ß-L-hexadeoxyuridylic acid were hydrolysed by SVP[305] at rates comparable to those reported for α-oligodeoxyribonucleotides.[282b] A trideca-ß-D-oligodeoxyribonucleotide, containing three consecutive ß-L-dC residues at the 5'-terminus, hybridized with a complementary and unmodified DNA sequence. The Tm of the hybrid was slightly lower than that of the native DNA duplex (ΔTm = 3 °C).[305] Given these properties, the application of mixed ß-(L)-D-oligodeoxyribonucleotides as potential antiviral agents should be further investigated.

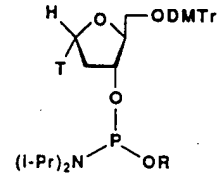
In this context, the preparation of the four ß-L-deoxyribonucleoside phosphoramidites **198a-d** and the solid-phase synthesis of the hexadeoxyribonucleotide ß-L-d(CGCGCG) have independently been described by Urata *et al.*[306] Consistent with the observations reported by others,[304,305] the oligomer was totally resistant to the nucleolytic activity of nuclease P1.[306]



194



195 B= T; R= Me; R'= H
196 = U; = Me; = H
197 = C^{bz}; = Me; = H
198a = T; = CH_2CH_2CN; R'= H
b = C^{bz} = CH_2CH_2CN; = H
c = A^{bz} = CH_2CH_2CN; = H
d = G^{Ib} = CH_2CH_2CN; = H
199 = U = CH_2CH_2CN; = $OSi(Me)_2Bu\text{-}t$



200a R= Me
b = CH_2CH_2CN

The synthesis and physicochemical properties of α-L- and ß-L-oligodeoxyribonucleotides covalently linked to an acridine derivative have been investigated by Asseline *et al.*[307a] The ß-L- and α-L-phosphoramidites **198a** and **200b** were prepared and applied to the solid-phase synthesis of octathymidylates having a phosphorothioate function at the 3'-terminus. These were then coupled with 2-methoxy-6-chloro-(ω-bromopentylamino)acridine and purified. The resulting oligonucleotide analogues were resistant to the 3'-exonucleolytic activity of SVP by virtue of the protection provided by the acridine moiety at the 3' terminus.[307a] The oligomers did not show degradation upon incubation with calf thymus 5'-exonuclease or with nuclease P1 for 72 h. By comparison, similarly synthesized α- and ß-D-oligothymidylates were almost completely hydrolysed upon treatment with calf thymus 5'-nuclease within 40 h and 7 min, respectively. Additionally, nuclease P1 digested the α-D-oligomer within 42 h.[307a] Both ß-L- and α-L-3'-acridinyl oligothymidylates did not interact with poly dA or poly

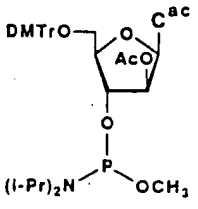
rA under various salt and temperature conditions. Conversely, ß-L- and α-L-tetradeoxyadenylates covalently attached to an acridine derivative, formed double and triple helices with poly rU or poly dT. These hybrids were considerably weaker than those obtained with the corresponding ß-D-tetradeoxyadenylate.[307a]

In contrast to ß-L-dU_{12}, the oligoribouridylic acid ß-L-rU_{12} prepared from the ribonucleoside phosphoramidite **199** strongly hybridized to poly rA (Tm = 38 °C in 1.0 M sodium chloride) relative to ß-D-rU_{12} (Tm = 40 °C).[307b] In addition, ß-L-rU_{12} was resistant to digestion with RNase A and whole cell extracts from L-cells for at least for 48 h and 4 h, respectively. Under similar conditions, ß-D-rU_{12} was completely hydrolyzed by RNase A within 30 sec while it took 2 h for the whole cell extracts to quantitatively hydrolyse the native polyribouridylate. Collectively, these findings indicate, that ß-(L)-RNA can form reasonably stable heterochiral duplexes with ß-D-RNA in parallel-stranded A-like conformations and may prove suitable for antisense experiments.[307b]

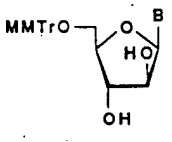
It is known that *ara*C is a potent antileukemic agent as well as an inhibitor of DNA replication.[308] It has been postulated that its cytotoxic effects resulted from its incorporation into DNA as a "fraudulent" nucleotide. The insertion of *ara*C at defined locations in synthetic oligonucleotides would therefore facilitate investigations regarding the molecular implication of *ara*C with DNA chain elongation, replication fidelity, and fragment ligation.

Beardsley *et al.*[309a] prepared the protected *ara*C phosphoramidite **201** which was incorporated at a specific location into a self-complementary oligonucleotide (d[CGCGAATT*ara*CGCG]) by usual solid-phase synthesis. The duplex resulting from the annealing of the modified oligonucleotide was slightly less stable than that of the duplex formed with the unmodified oligonucleotide (ΔTm = 4 °C) thereby indicating that the *ara*C-G base pairs did not significantly disturb the DNA duplex.[309a] The most striking effect was observed when *ara*C was located at the 3'-terminus of a DNA primer. All the polymerases tested (*E. coli* Pol I, T4 polymerase, AMV reverse transcriptase and HeLa cells Pol α_2) utilized *ara*C-terminated primer/template substrates very poorly.[309b] When compared to unmodified primers, the rate of incorporation of the next nucleotide was reduced by 100 fold, even though polymerases with associated (3'→5')-exonuclease activity preferentially excised *ara*CMP from the primer terminus prior to chain elongation.[309a,b] It is, therefore, difficult to argue that the inhibition of DNA synthesis was due to the slight instability observed with *ara*C-G base pairs. The mechanism whereby the incorporation of *ara*C into DNA inhibits DNA replication appears complex and remains to be elucidated.

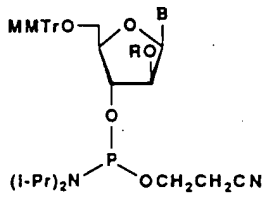
The synthesis of the arabinonucleoside phosphoramidites **203a-b** from the reaction of suitably protected arabinonucleosides (**202a-b**) with chloro-(2-cyanoethoxy)-*N,N*-diisopropylaminophosphine was reported by Damha *et al.*[310] It was pointed out that the phosphitylation reaction did not occur with complete regioselectivity, as 2'-*O*-phosphoramidites (*ca.* 3%) and 2',3'-bisphosphoramidites (5-10%) were also produced. The acetylation of **203a-b** led to the quantitative formation of **204a-b** which upon activation with 1*H*-tetrazole enabled the solid-phase synthesis of 5'-*ara*(UAUAUA).[310]



201



202a B= U
b = A^{bz}



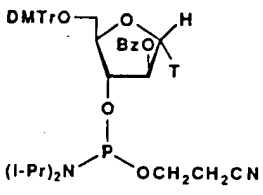
203a-b R= H
204a-b = Ac

Oligonucleotides containing 1'-α-D-arabinofuranosylthymines were synthesized by Adams *et al.*[311] The synthetic strategy consisted of the regioselective benzoylation of the 2'-OH function of 5'-*O*-DMTr-1'-α-D-arabinofuranosylthymine and the conversion of the resulting benzoate to the phosphoramidite

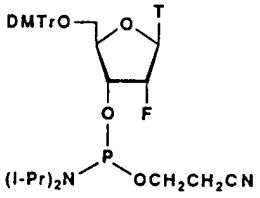
derivative **205**. The solid-phase synthesis of a pentadecathymidylate (α-*ara*T_{15}) from **205** was achieved according to a RNA synthesis protocol which led to coupling yields averaging 97%. The purified oligothymidylate was hybridized with d($C_3A_{15}C_3$) at near physiological conditions. The T*m* of the resulting hybrid was considerably lower than that observed with the native dT_{15} sequence (ΔTm = 25 °C). However, α-*ara*T_{15} and dT_{15} generated hybrids of similar stability with polyadenylic acid.[311] Thus, oligonucleotides containing 1'-α-D-arabinofuranosylthymines could be useful in antisense research.

Synthetic oligonucleotides having modified carbohydrates are of interest with respect to unusual conformational properties and stability to cellular nucleases. Particularly, the 2'-fluorothymidine phosphoramidite **206** has been applied to the solid-phase synthesis of complementary oligonucleotides (18-mers) containing the *Eco*RV recognition sequence GATATC.[312] The thermal denaturation of a modified duplex showed a significant decrease in stability relative to the unmodified duplex (ΔTm = 4.2 °C) when both thymidines of one strand of the recognition sequence were replaced with 2'-fluorothymidines. Such a duplex was cleaved by *Eco*RV at one-third the rate of the native hybrid, whereas a duplex having a 2'-fluorothymidine at the scissile linkage in each strand was digested at two-third the rate of an unmodified duplex. These data indicate that the increased resistance of these hybrids to cleavage by *Eco*RV emerged from the altered conformations of the duplexes resulting from the incorporation of 2'-fluorothymidines rather than the electronegative effect induced by the 2'-fluoro substituents.[312]

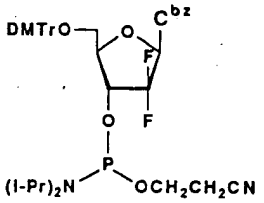
The incorporation of the 2',2'-difluoro-2'-deoxycytidine phosphoramidite **207** into oligodeoxyribonucleotides (19-mers) carrying recognition sequences for the restriction endonucleases *Kpn*I, *Bam*HI, *Hpa*II, and *Msp*I has also been reported.[313] It was shown that the insertion of 2',2'-difluoro-2'-deoxycytidine, at the scission sites, reduced the rates of cleavage with *Kpn*I and *Hpa*II by *ca.* 10% and 4%, respectively, relative to the rates obtained with unmodified duplexes. Furthermore, duplexes resulting from the single insertion of **207** exhibited lower thermostability than that of the unmodified duplexes (ΔTm = 2-4 °C). An even more pronounced thermal instability was observed, under similar conditions, with a duplex having one fluorinated deoxycytidine in each strand (ΔTm = 8 °C).[313] Altogether, the incorporation of 2',2'-difluoro-2'-deoxycytidine into DNA significantly affected duplex stability and, in few cases, slightly decreased the rate of endonucleolytic digestion.



205



206

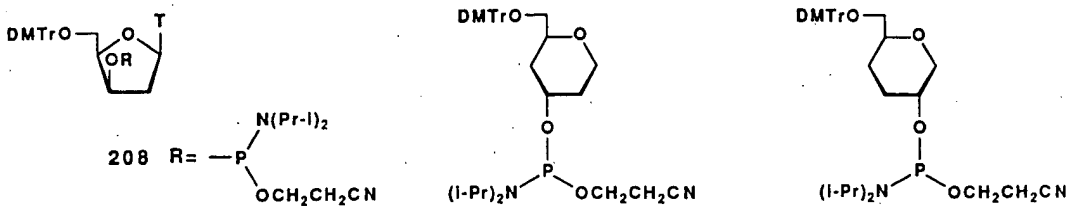


207

Rosemeyer and Seela[314] delineated the synthesis of oligonucleotides containing 1-(2'-deoxy-ß-D-xylofuranosyl)thymines. The deoxyxylonucleoside phosphoramidite **208** has been prepared and applied to the solid-phase synthesis of an oligothymidylate (13-mer) having twelve consecutive 1-(2'-deoxy-ß-D-xylofuranosyl)thymines. The modified oligothymidylate was resistant to CSP but was completely digested by SVP within 45 min at 35 °C.[314] The oligothymidylate analogue formed a stable complex with dA_{12} (T*m* = 36 °C) relative to that of the natural DNA duplex (T*m* = 43 °C). CD spectroscopy revealed that the modified oligothymidylate existed as a left-handed helical single-strand similar to the strands of Z-DNA.[314] In addition, the general pattern displayed by the CD spectrum of a complex composed of the oligodeoxyxylothymidylate and dA_{12} corresponded to that of Z-like DNA. These data implied that the hybridization of the single-stranded and right-handed helix dA_{12} with a left-handed and single-stranded oligodeoxyxylothymidylate resulted in a left-handed double-helix.[314]

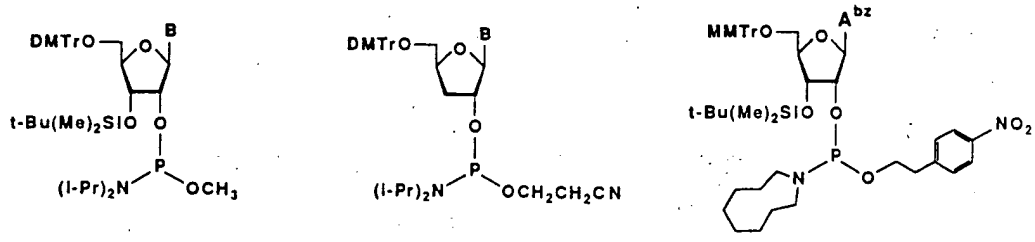
Oligonucleotides containing either 1-(2,4-dideoxy-ß-D-*erythro*-hexopyranosyl)thymines or 1-(3,4-dideoxy-ß-D-*erythro*-hexopyranosyl)thymines were synthesized from the respective phosphoramidites

209 and 210 on a solid support.[315] The double incorporation of 209 at each terminus of an oligothymidylate (13-mer) resulted in the formation of the most stable duplex with dA_{13} relative to the parent unmodified duplex. Furthermore, the modified oligothymidylate was slightly more resistant than the natural oligothymidylate to digestion with SVP. Conversely, modified oligothymidylates resulting from the double incorporation of the phosphoramidite 210 at each end of the oligomer provided strong resistance to degradation with SVP.[315a] Oligonucleotides modified by the incorporation of 209 are being further evaluated as antisense molecules.



3.9. (2'→5')-Oligonucleotides.

The ability of (2'→5')-oligoribonucleotides to form ordered structures is mostly unknown. To gain information about the association of these oligoribonucleotides, Kierzek *et al.*[316] described the preparation of the ribonucleoside phosphoramidites **211a-d** and the solid-phase synthesis of the self-complementary oligoribonucleotide (2'→5')-CGGCGCCG. The deprotected and purified oligomer produced, upon annealing, a complex having a T*m* of 46 °C at high ionic strength (1.0 M sodium chloride). This T*m* was considerably lower than that extrapolated for the same (3'→5')-oligonucleotidic sequence under similar conditions (T*m* = 79 °C). NMR analysis of the complex indicated the formation of an antiparrallel duplex.[316] In this context, (2'→5')-oligodeoxyribonucleotides have also been prepared from deoxyribonucleoside phosphoramidite precursors (**212a-d**).[317,318] Consistent with the findings of Kierzek *et al.*[316] some association between (2'→5')-dA_{12} and (2'→5')-dU_{12} occurred at high salt concentration (1 M sodium chloride) (T*m* = 22.8 °C). This association was, however, weaker than that observed with unmodified DNA strands under the same conditions (T*m* = 40.8 °C).[318] It has nonetheless been reported that the self-complementary (2'→5')-$d(AU)_6$ formed a hairpin structure having a higher T*m* value (39.3 °C) than that observed with the native $d(AU)_6$ oligomer (T*m* = 29.9 °C).[318] It is difficult to predict the usefulness of (2'→5')-oligonucleotides as antisense molecules since no thermostability data pertaining to (2'→5')-DNA or RNA/(3'→5')-RNA hybrids under physiological conditions have, as yet, been published.



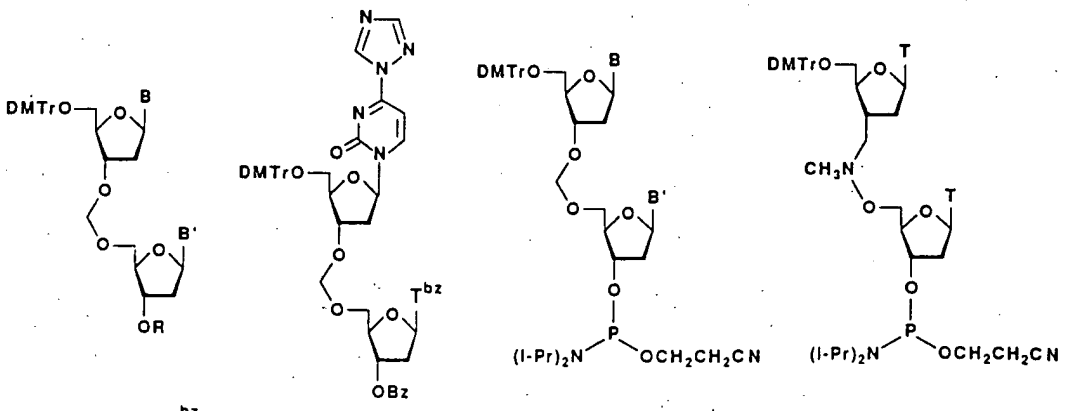
It has been known for some time that the (2'→5')-oligoadenylate pppA(2'→5')pA(2'→5')pA [2-5A] played an important role in the antiviral mechanism of interferon, and the regulation of cell growth and

differentiation.[319] In recent years, the synthesis of 2-5A analogues of has been undertaken to mechanistically probe its mode of action. The ribonucleoside phosphoramidite **213** has been particularly useful in the synthesis of oligoadenylates with (2'→5')-phosphorothioate linkages.[320a,b,g] In addition to providing resistance to nucleases, the inherent chirality of the phosphorothioate functions led, in the case of the trimeric 2-5A, to four diastereoisomers, which after separation, have demonstrated different abilities in binding to and activating RNase L from L929 extracts.[320c,d] In contrast, the diastereoisomers of the fully phosphorothioated (3'→5')-adenylate trimer did not bind to and did not activate RNase L, an enzyme involved in the interferon-induced antiviral and antiproliferative cascade.[320e] The phosphorothioate analogues of 2-5A also inhibited HIV-1 reverse transcriptase in viral lysates, and HIV-1 replication in MT-2 cells.[320f] These inhibitory effects represent an important function of (2'→5')-oligoadenylate analogues in the control of retrovirus replication.

3.10. Oligonucleotides Having Defined Unphosphorylated Internucleosidic Bridges.

In an effort to design oligonucleotide analogues as potential inhibitors of gene expresssion, the preparation of DNA segments having some of the native phosphodiester linkages replaced with enzymatically stable, achiral and uncharged methylene acetal bridges, has been reported.[321] Specifically, 5'-*O*-DMTr-N^3-benzoyl-3'-*O*-(4-pentenyloxymethyl)thymidine reacted with N^3-benzoyl-3'-*O*-methoxyacethyl thymidine and *N*-iodosuccinimide to give the dimer **214** in 80% yield.[321a,b] Following the selective removal of the methoxyacetyl group from **214** with catalytic amount of potassium *tert*-butoxide in dichloromethane-methanol (1:1). the 3'-OH function of the resulting dimer was phosphitylated with 2-cyanoethoxy-(*N,N*-diisopropylamino)chlorophosphine and *N,N*-diisopropyl-ethylamine (DIPEA) affording the dinucleoside phosphoramidite **218**. One or more T-CH_2T dimer(s) can be incorporated into an oligonucleotidic chain by the standard solid-phase phosphoramidite method. The coupling efficiency of the dinucleoside phosphoramidite **218** was *ca.* 95%.[321a,b]

Given the stability of N^3-unprotected thymidine to the iodonium-promoted coupling conditions, the synthesis of the dinucleoside (3'→5')-methylene acetal **215** was accomplished in excellent yields.[322] Treatment of **215** with excess phosphoryl tris-triazolide yielded the 4-triazolo derivative **217** which upon treatment with concentrated ammonium hydroxide and subsequent chemoselective benzoylation, afforded the dinucleoside (3'→5')-methylene acetal **216**. The phosphitylation of **216** with 2-cyanoethoxy-(*N,N*-diisopropylamino)chlorophosphine and DIPEA produced the dinucleoside phosphoramidite **219** in good yields.[322] This approach enabled the synthesis of DNA segments having (3'→5')-methylene acetal linked to cytosine nucleosides. Attempts to condense N^4-protected-3'-*O*-methylthiomethylene



214 B= B'= T^{bz};
R= methoxyacetyl
215 B= U; B'= T^{bz};
R= benzoyl
216 B= C^{bz}; B'= T; R= H

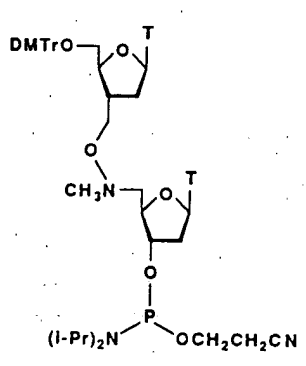
217

218 B= B'= T^{bz}
219 B= C^{bz}; B'= T

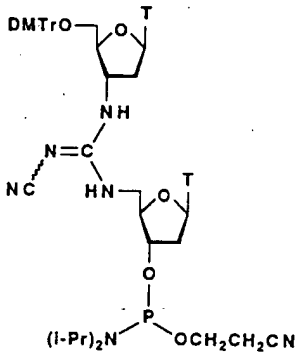
220

cytidine derivatives with an appropriate thymidine acceptor in the presence of *N*-iodosuccinimide and catalytic amounts of triflic acid, failed.[322]

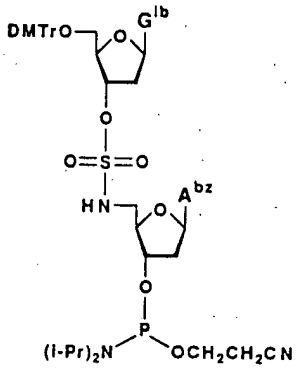
Along these lines, the oligodeoxyribonucleoside phosphoramidite **220** has been prepared and incorporated into oligonucleotides (15-18 mers) to assess the hybridization properties and nuclease resistance of the corresponding oligonucleotide analogues.[323a] The average coupling efficiency of **220** was *ca.* 99%. Hybridization studies revealed that the insertion of one to five modified internucleosidic linkages had little effect on the stability of duplexes composed of the oligonucleotide analogues and complementary RNA oligomers with respect to unmodified DNA/RNA duplexes. In addition, an hexadecaoligonucleotide containing five modified internucleosidic bridges had a full-length half-life of 16 h when incubated with HeLa cellular extracts. Under similar conditions, the unmodified oligomer exhibited a half-life of only 30 min.[323a] Interestingly, the incorporation of the dinucleoside phosphoramidite **221** into an hexadecaoligonucleotide produced an oligomer having a lower affinity for its complementary RNA sequence and increased sensitivity to nucleases relative to an oligonucleotide similarly modified by the insertion of **220**.[323b,c] Thus, oligonucleotides modified by the incorporation of **220** add to the repertoire of promising analogues for the inhibition of gene expression by antisense techniques.



221



222



223

The dimeric phosphoramidite **222** has been synthesized from 3'-amino-3'-deoxythymidine, *S,S*-dimethyl-*N*-cyanodithioiminocarbonate and 5'-amino-5'-deoxythymidine toward the development of uncharged, enzymatically stable, and achiral oligonucleotides capable of hybridizing with target sequences.[324] However, the incorporation of **222** into oligonucleotides and the physicochemical properties of these modified oligonucleotides have not, as yet, been reported.

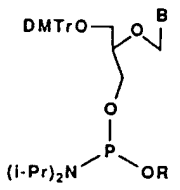
The sulfamate-linked dinucleoside phosphoramidite **223** has been prepared and singly incorporated into complementary oligonucleotides {d(GAGCTC[G*A]ATTCACTGGCCG) and d(CGGCCACT[G*A]ATTCGAGCTC)} containing an *Eco*RI recognition sequence.[325] Upon hybridization, the resulting duplex was slightly less stable than the parent unmodified duplex ($\Delta Tm = 3$ °C) indicating that the sulfamate linkage did not significantly affect the thermodynamic stability of the hybrid. It was also found that the sulfamate linkage was totally resistant to cleavage by both SVP and *Eco*RI.[325] Consequently, sulfamate-modified oligonucleotides may be useful for probing nuclease-DNA interactions or in antisense experiments for therapeutic applications.

3.11. Acyclic Oligonucleotide Analogues.

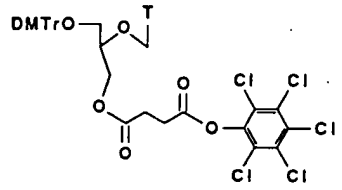
Acyclic analogues of naturally occurring deoxyribonucleosides derived from glycerol have been found active against a range of viral pathogens *in vitro* and *in vivo*.[326a,b] The incorporation of glyceronucleosides[326c] into oligonucleotides may be of interest, as these oligonucleotide analogues may also have antiviral activity. Usman *et al.*[326b] reported the synthesis of the glyceronucleoside

phosphoramidites **224a-b** and the reaction of LCAA-CPG support with **226**. The condensation of **224a-b** with the derivatized support yielded glycerooligonucleotides resisting to the nucleolytic activity of both SVP and CSP.[326b]

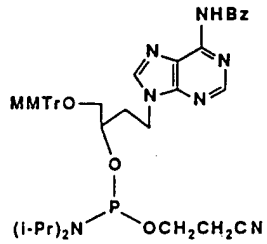
Oligonucleotide analogues containing glyceronucleoside residues at defined locations have been prepared by Schneider and Benner.[327] Typically, the glycerophosphoramidite **225** was inserted in oligonucleotides by solid-phase methods with a coupling efficiency of *ca.* 90%. Like a GT mismatch, each "flexible" nucleoside analogue decreased the T*m* of DNA duplexes by 9-15 °C.[327] It is, therefore, unlikely that oligonucleotides (*ca.* 15 bases) composed of such acyclic nucleosides could form stable duplex structures with complementary natural oligonucleotides in aqueous solution.



224a B = T; R = Me
b = A^{bz}; = Me
225 = T; = CH_2CH_2CN



226



227

Augustyns *et al.*[328] reported the incorporation of (*S*)-9-(3,4-dihydroxybutyl)adenine, as a nucleoside substitute, in the synthesis of dimers. The acyclic phosphoramidite **227** was coupled with 2'-deoxyadenosine to test the applicability of standard DNA synthesis protocols and the sensitivity of the modified dimers to nucleases. A*p(dA), where A* represents the acyclic nucleoside, was 50% hydrolysed by SVP within 129 min but was stable to S1 nuclease and bovine spleen phosphodiesterase (BSP). Alternatively, (dA)pA* was digested to the extent of 50% with S1 nuclease, SVP, and BSP within 8 min, 568 min, and 22 min, respectively. Under the same conditions and in the same order, the native d(ApA) was 50% degraded within 17 min, 26 min, and 4 min, respectively. Incidentally, the fully modified dimer A*pA* was stable to the nucleolytic activity of these enzymes.[328] The acyclic phosphoramidite **227** has also been incorporated at selected positions into oligonucleotides during solid-phase synthesis. An oligodeoxyriboadenylate ($A^*p[dAp]_{11}A^*$) having one acyclic analogue at each terminus was hydrolysed by SVP at a rate seventeen times slower than that of dA_{13}. The modified oligonucleotide formed a duplex with dT_{13} exhibiting a slightly lower T*m* than that of the natural duplex dA_{13}/dT_{13} (ΔTm = 3 °C).[328] Because of demonstrated stability to nucleases and retention of base-pairing properties, the utilization of similar oligonucleotide analogues should be considered when performing antisense studies.

CONCLUDING REMARKS

While nucleosidic phosphoramidite derivatives have tremendously facilitated the automated synthesis of oligonucleotides,[1] modified nucleosidic and non-nucleosidic phosphoramidites have enabled the functionalization of oligonucleotides for biomedical applications.[2] Furthermore, the synthesis of modified oligonucleotides *via* phosphoramidite precursors has played a strategic role in the study of protein-DNA interactions, DNA/RNA recognition, and the control of gene expression. These applications, among others, have been reviewed in this Report. Nucleosidic phosphoramidites have also

been applied to the synthesis of "branched" RNA oligonucleotides to gain insight into the chemical processing (splicing) of pre-mRNA, a prerequisite event in the proper expression of eukaryotic genes. Alternatively, phosphoramidite derivatives have served in the phosphorylation of biomolecules such as, for example, phosphopeptides and *myo*-inositols. It is generally accepted that protein phosphorylation and cell signalling constitute the cornerstone of an ubiquitous transduction mechanism known to regulate a large array of cellular processes including metabolism, secretion, contraction, neural activity and cell proliferation. These applications along with the synthesis of "branched" RNA oligonucleotides, and recent advances pertaining to the structure and function of catalytic RNA molecules will be the focus of a future Report.

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IMPROVED SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES

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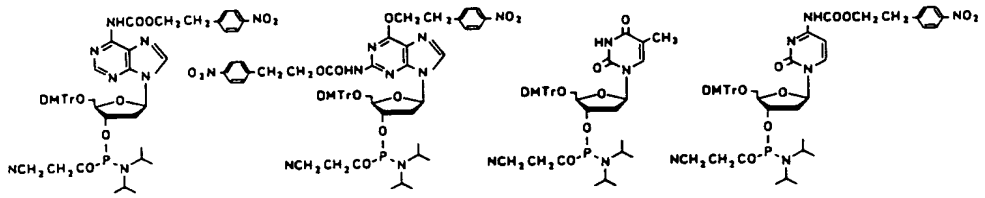
Abstract. The design of a new polymer support in combination with the well experienced ß-eliminating protecting groups offers an improved approach for automated oligonucleotide synthesis. The advantages are notorious by the possibility of preparing fully deblocked still support-bound oligomers, which result on final liberation in high yield, easy isolation and high purity. This approach also reveals some options, which have so far not been realized in the field of solid phase synthesis of nucleic acid fragments.

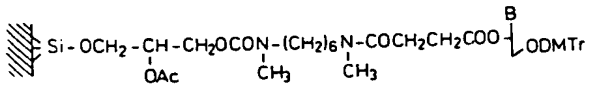
The development of the phosphoramidite methodology[1,2] can be regarded as a break-through in chemical solid phase supported oligodeoxyribonucleotide synthesis due to a high degree of automatisation of the chain elongation process. A great number of variations of the original method has been published, dealing mainly with changes in the blocking group patterns of the aglycone, sugar and phosphite moiety of the monomeric building blocks as well as alterations of the material of the solid matrix and the design of the spacer connecting the starting nucleoside covalently with the support. The excellent results obtained on application of the p-nitrophenylethyl (NPE) and p-nitrophenylethoxycarbonyl (NPEOC) blocking groups[3] in solution synthesis of oligonucleotides[4,5] prompted us to develop a solid phase synthesis strategy, in which all synthetic as well as the deprotection steps could be done while the oligonucleotide is still attached to the support allowing an easy removal of any by-products and excess of reagents by simple washing and filtration[6].

The new strategy afforded in the first place the synthesis of a new set of 3'-(2-cyanoethyl)-N,N-diisopropylamido-phosphites differing from the commonly used and commercially available building blocks by applying the p-nitrophenylethoxycarbonyl (NPEOC) group for amino protection instead of the benzoyl and isobutyryl group respectively and the additional blocking of the amide function of 2'-deoxyguanosine by the p-nitrophenylethyl (NPE) residue.

The syntheses have been achieved in the usual manner by protecting first the aglycone moiety[3] followed by 5'-O-dimethoxytritylation and subsequent phosphitylation with 2-cyanoethyl-



bis(N,N-diisopropylamino)-phosphane[7] under tetrazole catalysis. Furthermore a new type of modified solid support material had to be prepared since the ester function of the succinyl spacer of the commercially available LCAA-CGP supports is not stable under the deprotection conditions using DBU or MTBD in aprotic solvents. In order to overcome the intramolecular nucleophilic attack of the neighbouring deprotonated amide group[8,9] dihydroxypropyl-CPG (500A and 1400A pores) beads were reacted first with N,N'-carbonyldiimidazole and then with 1,6-bis-methylaminohexane as an aliphatic secondary amine spacer. Final coupling with the appropriately protected 2'-deoxynucleoside-3'-O-succinates and subsequent capping of the free hydroxyl groups of the matrix led to loadings of the anchored starting nucleoside of 20 micromoles per gram support.

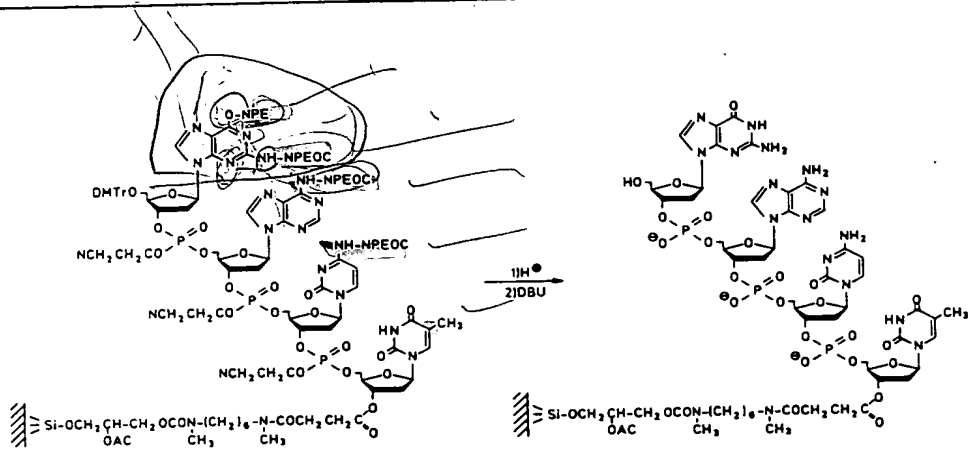


This support was proved to be completely stable under the deblocking conditions used for the NPE and NPEOC groups, while cleavage from the matrix can be achieved normally under hydrolytic conditions in conc. ammonia solution in less than two hours.

Several oligodeoxyribonucleotides have been synthesized in a DNA-synthesizer (Applied Biosystem Model 380 B) applying both acyl and NPE/NPEOC protected phosphoramidites for comparative studies. The chain elongation cycle was only slightly modified in using a 25 fold excess of the phosphoramidite reactant in regard to the support-bound 5'-OH component and a total condensation time of 60 seconds. The yield of each coupling step was determined by the colorimetric assay of the released dimethoxytrityl cation. The obtained yields indicate that the NPE/NPEOC protected phosphoramidites give similar or even better results as the common acyl blocked analogues.

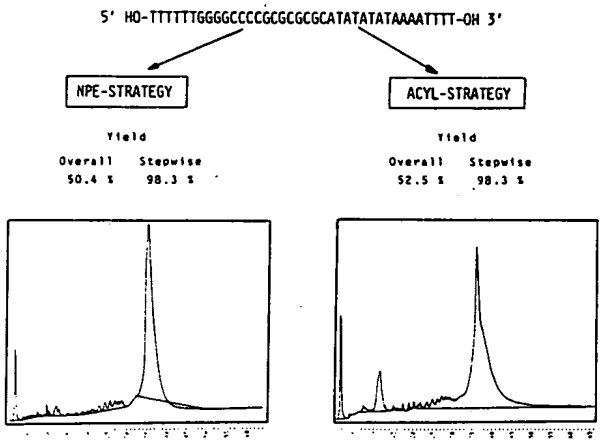
Tab. 1 - Results of comparative DNA-Syntheses

Sequence	Acyl-Strategy Overall %	Acyl-Strategy / Stepwise %	NPE/NPEOC Strategy Overall %	NPE/NPEOC Strategy / Stepwise %
HO-TTTTTTGGGGCCCCCGCGCGCGATATATATAAAATTTTTT-OH	52.5	98.3	50.4	98.2
HO-TAAAAACCCCGGGGCGCGCGCGTATATATATTTTAAAAAT-OH	53.4	98.4	61.0	98.9
HO-TTTTTTGGGGCCCCCGCGCGCGATATATATAAAATTTTTT) HO-TAAAAATTTTATATATATGCGCGCGCGGGGCCCCAAAAAT			48.6	99.8
HO-ATGCATGCGGGGTTTTATGCGCATAAAACCCCGCATGCAT-OH			55.6	98.3



The big advantage of the NPE/NPEOC-strategy became obvious during the deprotection procedure. Total deblocking of a synthesized oligonucleotide was achieved first by removal of the 5'-O-dimethoxytrityl group automatically by choosing the option "trityl off" in the synthesis program. The cyanoethyl as well as the NPE and NPEOC groups were then eliminated by treatment with 0.5 M DBU in acetonitrile during 6 hours at room temp. on the synthesizer. This way it is possible to obtain totally deprotected oligonucleotides, which are still attached to the solid support in high yields, demonstrating the superiority of this approach over the method of Seliger[10]. So far only less than 5 % of the oligonucleotide chain from the support was detected under the deprotection conditions on applying the "trityl on" program. Cleavage of the support-bound oligonucleotide finally was achieved by conc. aqueous ammonia by the common automated procedure to yield the crude product free of any by-products of the previous synthesis/deprotection steps. This material was then analysed by anion ex-

change HPLC on a Nucleogen 60-7 DEAE column[11] using a LiCl gradient (0-1 M) and compared with the same sequence material from the acyl strategy.



The improvements of this new approach have to be seen in a time-saving easy isolation procedure, better yields and higher purities of the oligonucleotides. Furthermore oligonucleotides containing modified base moieties will also be available on these lines.

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